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Address of the President  
Sir Robert Robinson, at the  
Anniversary Meeting, 30 November 1948

On behalf of the Fellows of the Royal Society the following telegram was sent on 16 November to His Majesty the King:

‘We your loyal and dutiful subjects the President, Council and Fellows of the Royal Society of London beg leave to offer our respectful and sincere felicitations on the birth of the Prince.’

A message was also sent to Her Royal Highness The Princess Elizabeth as follows:

‘The President, Council and Fellows of the Royal Society of London send their respectful and sincere congratulations to Your Royal Highness on the happy event of the birth of the Prince.’

We have received gracious acknowledgements in the following terms:

‘Please convey to the Council and Fellows of the Royal Society the sincere thanks of the Queen and myself for their kind message on the birth of our grandchild.

GEORGE R.’

‘We are most grateful for your kind message of congratulations.’

ELIZABETH AND PHILIP.’

*Award of Medals, 1948*

The COPLEY MEDAL is awarded to Professor ARCHIBALD VIVIAN HILL, C.H., for his outstanding contributions to the physiology of muscular processes.

Towards the end of the nineteenth century physiologists discerned a field of investigation of the physical and chemical phenomena involved in the characteristic activity of muscle and nerve, but the questions propounded could receive no satisfactory answers, chiefly owing to the lack of adequate methods. Hill, during the last thirty-five years, has applied profound mathematical skill and a flair for the design and management of delicate instruments to the provision of a sound basis for experimental and theoretical advance. In his hands the thermopile was developed into an instrument of great precision and delicacy, and conditions for its use in relation to the material studied were established so as to provide significant results.

The work on heat production in muscle has led to a picture within a framework which can accommodate many contemporary biochemical studies in a harmonious fashion. Attention has constantly been paid to ensuring the thermodynamic soundness of the systems suggested. Moreover, he was able to exploit some of his findings on isolated muscle in the course of a study of the dynamics of muscular contraction in the living body, including that in the human subject.

A natural extension of Hill's researches has enabled him to contribute notably to our knowledge of the heat production and excitability of nerve. Previous workers failed to detect any heat production in nerve tissues, but Hill demonstrated the definite thermal changes that are associated with the passage of impulses. These were found to be so small that no one-way contemporary chemical reaction could account for them. It would be necessary to postulate reversible changes which proceed in both directions. This was a main factor in the elaboration of the modern view that the nerve impulse results in an altered state of polarization of the essential surface membranes. The heat change which occurs after the passage of the impulses was found to be small but definite, and was related to the energy expended in the recovery of the membrane.

Hill has made contributions of great distinction in other fields, and among other topics investigated by him is that of the physical chemistry of the oxyhaemoglobin equilibrium in the blood.

Hill has been a source of inspiration to many pupils, collaborators and colleagues and has founded a school of research. His eminent services to physiology have been possible as the result of a happy combination of wide basic knowledge of the physical sciences, unusual skill in the design and execution of experiments, a philosophic outlook, and above all the curiosity and ambition to attack some of the most difficult problems in biophysics and biochemistry.

The RUMFORD MEDAL is awarded to Professor FRANZ EUGEN SIMON, C.B.E., for his distinguished researches on the properties of matter at low temperatures.

The school of low-temperature physics which Simon has created at Oxford is one of the leading centres of such research in the world and has achieved highly important results in the spheres of both theory and practice.

His great knowledge of thermodynamics was brought into service in the development of extremely simple methods for reaching the temperatures of liquid helium in the laboratory. He realized that at low temperatures adiabatic expansion of helium compressed into a small reservoir would absorb sufficient heat to liquefy the bulk of the gas and to cool the container and specimens under examination to liquid helium temperatures. Thus, provided reasonable supplies of liquid air are available, it becomes possible for any laboratory to carry out investigations at the lowest attainable temperatures with equipment which is inexpensive and simple to operate. Many other contributions to technique in this field have been made by Simon and his co-workers.

With the aid of these new or improved methods many interesting observations, with a bearing on fundamental knowledge, have been made. Outstanding problems in superconductivity, especially the role played by impurities, have been solved, and the 'freezing out' of magnetic flux has been explained. The peculiar properties of liquid helium II and the transition to this state have been investigated intensively. The thermodynamics of the approach to absolute zero has been stated very clearly by Simon and progress has been made in new methods for reaching this temperature. More recently, extended equipment has facilitated the study of the properties of atomic nuclei oriented in magnetic fields.



Simon has made significant contributions to the elaboration of devices for the separation of isotopes and to the design of plants in which thermodynamic considerations are of predominating importance.

He has aptly illustrated the thesis that, in the world of modern physics, discovery and the progress of theory follow surely on the introduction of new and powerful tools of research.

A ROYAL MEDAL is awarded to Professor HAROLD JEFFREYS for his fundamental investigations in theoretical geomechanics.

Much of Jeffreys's well-known book *The Earth* (1924, 1929) is based on his own work, and this is especially true of the section on near earthquakes. However, the book owes its unique position not only to these original contributions but also to the skill and insight shown in the building up of a self-consistent picture of the earth from the very diverse and apparently contradictory materials available at the time when it was written. Its great influence has been due to the width of interest and the critical skill as well as to the depth of learning of its author.

During the thirties Jeffreys's main interest was in seismology. In a series of papers he has discussed the available observations in detail and reduced the errors in the seismological time-tables by a factor of 10 or more. This work has had an important effect on our knowledge of the earth's interior, and has yielded numerical information of surprising accuracy, and, for example, the radius of the earth's central core is now known with an accuracy of a few kilometres.

Some of his early papers and the book *Scientific Inference* show an interest in the philosophical basis of the scientific method and in the theory of errors. This was intensified by the difficulties he met in adequately treating the seismological travel times, to which orthodox 'least square' methods are inapplicable. The discussion of these observations led to a series of papers on significance tests, and, finally, to a book, *The Theory of Probability*, in which he discusses *de novo* the whole problem of the relation between observation and 'physical quantities'. The departures from orthodoxy have not proved universally acceptable, but it is generally agreed that Jeffreys has made an important contribution to a subject on which the last word has not yet been said.

Other topics to which Jeffreys has made material contributions are pure mathematics, the origin of the moon, the constitution of the planets, hydro- and aerodynamics. In particular, he gave the first mathematical discussion of the theory of aerofoils of finite thickness.

Other less relevant examples of the catholic interests of the Plumian Professor of Astronomy and Natural Philosophy could be cited. Whether as a specialized applied mathematician or as a teacher and leader of research, the eminent services he has rendered are most widely recognized.

A ROYAL MEDAL is awarded to Professor JAMES GRAY, C.B.E., for his distinguished work on the mechanism of posture and locomotion in vertebrate and other animals.

In 1933 Gray, by the use of cinematography, analyzed the forces involved in the progression and turning of fish with long and short bodies. The inquiry reached its

full development during the last ten years with the extension of these studies to vertebrates generally. In 1936 he discussed the swimming of dolphins, raising the hydrodynamical problem of the type of waterflow past an undulating body. Later he analyzed the locomotion of Amphibia and of snakes. The results of this part of the work led to a discussion of the mechanics of the tetrapod skeleton which was fundamental and placed an important part of the comparative anatomy of vertebrate animals on a functional basis for the first time. His work shows that the whole body of a tetrapod must be considered as a single functional unit, changes in the tension of one muscle being accompanied by a demonstrable pattern of changes in other muscles. Gray analyzed and described the mechanical problems solved by tetrapods standing and moving over ground of varying slope and smoothness; he has also analyzed the neuro-muscular phenomena responsible for the evident co-ordination of action. He showed the importance of peripheral reflexes in the initiation and maintenance of swimming movements in fish. This led him to the problem of the origin of locomotory patterns. His experiments on the neuro-muscular system of annelids showed the major importance of patterns of peripheral stimuli.

This work, summarized in a Croonian Lecture, was later greatly extended by demonstration of the importance of peripheral reflexes in the locomotor activities of toads. It was found that there was no positive evidence for the existence of central rhythm in these animals, while, on the other hand, the proprioceptive reflexes arising from the muscles provide an adequate basis for locomotor action.

Gray's work is responsible for an important change in the direction of research in the comparative anatomy of vertebrates. Before its appearance skeletal structure was examined in extraordinary detail, and yet little or no attention was paid to musculature and to the relation of structure to the whole system in action. More than any other, Gray has shown that comparative anatomy is certainly not a dead science.

The DAVY MEDAL is awarded to Professor EDMUND LANGLEY HIRST for his distinguished contributions to the chemistry of the carbohydrates.

The first demonstration of the six-membered ring structure of an aldose derivative was offered by Hirst & Purves in their study of methylxyloside which was published in 1923. Since then, in collaboration with Sir Norman Haworth over a number of years and independently, Hirst played a great part in the establishment of fundamentals in the field of carbohydrate structure. His prolific experimentation is characterized by the highest accuracy and has afforded conclusive answers to many questions of importance.

Of his joint work in the Birmingham School the following must be cited: the recognition of pyranose and furanose forms of the simple sugars, the isolation of a crystalline furanoside for the first time, and the determination of the constitution of sucrose and other disaccharides.

His independent work on optical rotatory dispersion in the carbohydrate derivatives introduced new methods of attack of the constitutional problems which were of immense service in his later work on the constitution of vitamin C. In that investigation of outstanding merit he determined the structure of ascorbic acid and

studied a large number of related products. In 1933 he shared in the synthesis of ascorbic acid and in the study of the physiological activity of the synthetic vitamin.

His researches on the polysaccharides have been conspicuously successful over a wide field, largely because of his development of new methods of end-group assay in the determination of structure. He has applied these methods successfully to starch, glycogen and other polysaccharides, and to him we owe many advances in our knowledge of the nature of alginic acid, the plant gums and pectic substances.

The regularities and irregularities that have been disclosed have been of equal interest and unexpectedness.

Hirst has been a pioneer in unravelling the complexities of carbohydrate architecture, and to him must be attributed much of our present knowledge of this group of such great biological significance.

THE DARWIN MEDAL is awarded to PROFESSOR RONALD AYLMER FISHER for his distinguished contributions to the study of biological evolution.

A general principle that is consistently developed in Professor Fisher's writings is that the course of evolution is not controlled by mutation but by selection operating upon the heritable variability which Mendelian recombination supplies.

In 1928 he was the first to suggest that dominance is the product of selection operating in the gene complex of the organism. This theory he tested and confirmed experimentally with domestic poultry, and later with mice.

The implications of his concept extend beyond the dominance phenomena, and underlie the whole theory of the modification of genic effects through selection acting on the gene complex. This enables a character, even when under unifactorial control, to be adjusted to the needs of the organism. To Darwin, selection was a mechanism for rejecting the bad and conserving the good; it is largely to Fisher that we owe the view that it may mitigate the bad and enhance the good effects of the hereditary units.

The studies of Fisher on abundance and variability, the first of them undertaken in collaboration, have provided means for proving in general terms the proposition, implicit in the modern concept of evolution, that genes having minute favourable effects are in process of being spread through populations and are bringing about evolutionary change at the present time.

Fisher's methods of population analysis, developed from 1930 onwards, have made it possible to study the numerical aspect of animal communities with an exactitude previously unattainable.

The special opportunities for evolutionary study presented by polymorphism have always attracted Fisher, and he has repeatedly analyzed the balance of selective agencies which may be involved. This point of view is apparent in his extensive work on human serology. He has also studied a polymorphism which had especially interested Darwin, that of the heterostyled plants, and he has extended the subject to elaborate the general theory of crossing-over in polyploids: a pioneer work of wide application.

Fisher's great contributions to statistics and to experimental design have provided tools now deemed essential to the quantitative biologist. But they are fundamental

also to the experimental studies of evolution which have become so important a feature of modern biological research.

The HUGHES MEDAL is awarded to SIR ROBERT WATSON-WATT, C.B., for his pioneer researches in radiotelegraphy.

Watson-Watt has been a leader in the field of radio research since the early nineteen-twenties. He began to work on the subject of atmospherics in 1915, paying attention at first to the subject of the direction of arrival. He published the first English studies on this subject, establishing the 'cum-solar' swing of the place of origin throughout the day. He developed the cathode-ray direction-finder as a means for finding the direction of arrival of individual atmospherics. This proved of immense value in the location of thunderstorms during the war. With a number of collaborators he later studied the wave form of atmospherics.

He has been the leading exponent of the use of the cathode-ray oscillograph for a great variety of purposes in physical research.

Watson-Watt was the leader of the earliest British work on radar, or radiolocation as it was then called. Although the basic principles were known, enormous technical difficulties had to be overcome before radar could be developed in the form of an operational instrument. That these difficulties were in fact overcome was due, in this country, more to Sir Robert Watson-Watt than to any other man. He is now turning with equal insistence to the peacetime applications of radar, especially in the service of civil aviation.

I wish to thank Fellows of the Society and members of Council for assistance in preparing notes on the achievements of the Medallists.

Sir Alfred Egerton now lays down his office as Physical Secretary of the Society. During the past ten years he has shown a deep concern for the Society's welfare and the utmost willingness to undertake onerous duties.

I would like to remind you of some of his many activities. In the early days of 1939 he was chiefly instrumental in the creation of a Central Register of Scientists. The significance of this action by the Society was by no means exhausted by the cessation of hostilities. Almost in the first month of the war he took a leading part in persuading the War Cabinet to set up a Scientific Advisory Committee. These efforts, after initial setbacks, succeeded in October 1940, and the Committee functioned usefully during the war period and was the forerunner of the present Advisory Committee on Scientific Policy. I cannot deal at length with his services in a more personal capacity, for example, to the Ministry of Fuel and Power, but we recall with gratitude his interest in the reorganization of the Gassiot Committee and in the survey, which the Society conducted, of the needs of fundamental science after the war. He has been an ever-welcome guest overseas, and has a distinguished record as an ambassador of science. His great services to the Empire Scientific Conference of 1946 and its offspring, the Scientific Information Conference of the present year, are fresh in our memory. He worked zealously, carefully and effectively—an unusual and difficult combination—and the results will benefit every part of the Commonwealth in which scientific activity exists.

Sir Alfred will continue to act as virtual representative of the Society in several important capacities; if I may mention just one as an example, it may be as a member of the Scientific Advisory Council for the Festival of Britain 1951.

We shall not be able to take so much advantage of his habitual generosity as in the past, but, in case of need, we shall certainly seek his help and advice.

You have just elected Professor David Brunt as the Physical Secretary of the Society. To him we offer the most cordial welcome and good wishes coupled with thanks for his willingness to assume the duties and responsibilities of the office.

One of the most valuable of the traditions of the Society is our claim to exercise initiative when the circumstances suggest a new departure. So far as I am aware it has not previously been thought desirable to designate any person as Honorary Librarian to the Society, but the Council felt that the present Chairman of the Library Committee had given services which were probably without precedent. Professor Andrade's knowledge of the older scientific literature is unrivalled and has been freely available to the great advantage of the Society.

The retirement of Mr H. W. Robinson as Librarian occurred at the end of March of this year. He joined the Staff in 1902 and assisted the chief clerk and the Librarian; in 1910 he became Library Assistant and in 1930 Assistant Librarian. He has been Librarian since 1935. His work for the Society and its Library has been invaluable, and we may recall with special gratitude the care he showed in the safe bestowal of our treasures in the time of danger. An earnest student of the history of science, he has paid special attention to the study of Robert Hooke on whose life and work he is a recognized authority. In expressing our gratitude for his devoted labours, we note with pleasure that Mr Robinson will continue to assist Professor Turnbull in editing the Newton Letters.

The outstanding event of the past year in connexion with the Society has been the Scientific Information Conference. We welcomed delegates from the countries of the Commonwealth and also from the National Academy of Sciences of the United States, and from U.N.E.S.C.O. It can thus be claimed that the whole of the English-speaking scientific world was well represented. More than half of all scientific literature is written in English, and in making such a comprehensive review of scientific information services, I think the Society may be judged to have taken a timely initiative. We were gratified by the interest shown by Fellows and the time and energy so freely given by them to ensure the success of the Conference.

Your Council received from the Conference many recommendations dealing with the publication of papers reporting original work, abstracting services, reviews and annual reports, library services, classification and indexing, and other relevant matters. It has been decided to implement these, as far as proves possible, with the aid of a Treasury grant, and for this purpose the Council has set up an Information Services Committee which has just held its first meeting. I believe that an energetic follow-up of the recommendations will result in real progress. The value of the Conference itself, irrespective of these recommendations, should not be underestimated. Divergent points of view were advocated, and many of the difficulties besetting the free and rapid communication of new knowledge were at least

recognized as the result of discussion and, in many cases, were removed. The problem is a complex one, and its solution demands both due regard for traditional methods and an acute appreciation of novel techniques.

Having given this lead the Society will now be expected to play an increasingly important part, nationally and internationally, in improving scientific information services.

It is doubtful whether the general progress of science has ever been more impressive than at the present time, and this fact emphasizes the importance of adequate outlets for the publication of the great volume of original work that is being poured out. Formidable archives are undoubtedly accumulating. To meet the demand many new journals have been founded and here is a short list of them. *Agricultural Chemicals*, Baltimore, 1946; *British Journal of Pharmacology and Chemotherapy*, U.K., 1946; *Hungarica Acta Chimica*, Budapest, 1946; *Journal of Colloid Science*, N.Y., 1946; *Journal of Polymer Science*, N.Y., 1946; *Zeitschrift für Naturforschung*, Wiesbaden, 1946; *Acta Chemica Scandinavica*, 1947, Chem. Soc. Denmark, Finland, Norway and Sweden; *Analytica Chimica Acta*, N.Y., 1947; *Biochemica et Biophysica Acta*, N.Y. 1947; *Die Makromolekulare Chemie*, Basle, 1947; *Food Technology*, N.Y., 1947; *Heredity*, London, 1947; *Journal of Glaciology*, U.K., 1947; *Nucleonics*, N.Y., 1947; *Acta Crystallographica*, U.K., 1948; *Acta Physica Austriaca*, Vienna, 1948; *Annali di Geofisica*, Rome, 1948; *Australian Journal of Scientific Research*, Australia, 1948; *Deutsche Hydrographische Zeitschrift*, Hamburg, 1948. Furthermore, the Scientific Reviews are constantly being extended in scope: one can even be thankful that they show a tendency to overlap. *Nature*, playing a more important part than ever before, is now supplemented by *Research*, whilst *Endeavour*, *Science Progress* and *Discovery* worthily maintain our reputation in the field of popular exposition. The press is far better informed on scientific affairs than was the case a few years ago, and we can anticipate useful autocatalysis in the reaction between public appreciation and public information.

A year ago I mentioned the formation of a committee to study the means whereby adequate accommodation for the scientific societies may be made available, and tactful reference to its activities will be found in the Report of Council. Without being too indiscreet it is possible to provide some further information on a subject of great interest to the Fellows of the Society. In the first place the Scientific Accommodation Committee has so far considered only the long-term problem, and it is matter for congratulation that representatives of so many interested parties reached full agreement on this aspect. It was unanimously agreed that the institution of a Science Centre would provide the best solution. Naturally everybody would like to know where that will be and exactly what the proposal involves. In regard to the first point a specific suggestion is being explored, and we hope that it will soon be possible to announce a definite outcome such that a suitable site will be allocated for the eventual creation of a worthy science centre. It is unlikely that this site will please everybody, but we have reason to believe that a large majority

will welcome it in view of the many advantages secured. In any case we have the assurance that favourable consideration will be given by Ministers to transference to a better site, should such be found.

The course of the discussion showed that the co-operation of several scientific societies depended on that of the Royal Society, and your representatives accepted the flattering implications, though not without some hesitation. To speak directly: that means willingness, if necessary in the general interest, to leave Burlington House and to function as the heart of the Science Centre located elsewhere.

The elaboration of so far-reaching a scheme is not at all a simple matter and involves polypartite negotiations and agreements. The Fellows of the Society will be directly consulted on the major issue just so soon as it can be put to them in a precise form.

One possibly related matter may be adumbrated at this early stage, so that we may think about it.

The Report of Council mentions the reconstitution of a 'Rutherford Memorial Committee' which has various schemes under consideration. At the last meeting of this Committee I ventured to suggest that, as one aspect of the Memorial, a 'Rutherford Hall' of noble design should form a part of the Science Centre, and this was given general approval by the Committee and later by Council.

Unfortunately, I must now turn from these roseate dreams of future glory to an incident of the present which has rudely disturbed our peace of mind. It is possible that our information is incomplete, or even inaccurate, but it is probable that we have ascertained the more important facts of the case.

I refer to the report that eminent Russian biologists have been constrained to subscribe to interpretations of the data in the field of genetics which they had previously rejected, or perhaps had thought unworthy of serious consideration. According to *Pravda* 'The Academy of Sciences forgot that the most important Principle in Science is the Party Principle'. That is a forthright declaration which leaves little scope for ambiguity. The incident is evidently of political rather than of scientific importance, and the Royal Society is not concerned with politics.

We regret that the Academy of Sciences of Moscow has broken off its long correspondence with us. We trust that the new conditions will not seriously impede the advance of biological science for which such qualifications as 'Western' are as irrelevant as they would be for a multiplication table. We impute no blame and express no opinion as a body, but that does not mean that we must take no cognizance of the occurrence which may have some lessons for us, at least by way of analogy. For example, we may observe that Governments are not infallible, yet must be obeyed. This reflection should make us more than ever alert to preserve intact the prized freedom of science in our own domain. Actually no direct attack is likely here, and should the unexpected happen it will certainly not be along the lines of compelling us to espouse some particular scientific theory or doctrine. Conceivably it could take the more subtle form of control of the character and direction of our scientific work. There is immediate danger in the current deprecation of fundamental research, not of course absolutely, but relatively, in comparison with

technological applications. I hope it will be the opinion of all Fellows that the Royal Society should take a leading part in upholding our ideals and in clarifying ideas on these topics and particularly in insisting on the vital role which the highest kind of disinterested investigation must play in the life of the community. It is certainly not sufficiently realized that the body scientific can only flourish when all its organs are in a healthy condition. As in a biological equilibrium there is a natural interdependence between pure and applied research. Pure science is fertilized by the advance of technology and vice versa. It would be quite consistent, though lamentable, to take up the position that we will have no more research at all and devote our energies to the exploitation of present knowledge. But it is impossible to dissect the elements of real progress. If we isolate one of the limbs of the organism it will not grow and will soon die.

I do not propose to add more than brief comment on new scientific discovery. It will be conceded that the great privilege of addressing the Fellows of the Royal Society is not without its own peculiar embarrassments. For example, I have recently delivered two lectures summarizing results of many years in my own field of work. If I venture on other ground I shall be speaking as an amateur to some of the leading specialists in the world.

Without attempting any kind of assessment of values, or any completeness of survey, I will mention a few things that have been brought to my notice in recent months, often as the result of casual conversation.

Two years ago I referred to the very great value of spectroscopic methods for the study of complex molecules of organic compounds. Since that time there have been striking applications of both ultra-violet and infra-red absorption measurements which have solved some otherwise intractable problems. But one difficulty that threatened to impede progress was the management of minute quantities of material, especially in the solid state.

Dr C. R. Burch, Warren Research Fellow, has built a reflecting microscope which is perfectly achromatic, and this has now been harnessed to an ultra-violet spectrometer by Barer, Holiday and Jope and to an infra-red spectrometer by Thompson, Barer and Cole. In the latter case the spectral range from 1 to  $14\mu$  can be covered, and the results are identical with those obtained with larger specimens in more usual apparatus.

The spectra of single biological cells or single crystals weighing less than  $10^{-7}$  g. have been measured. Polarized infra-red radiation has also been used, and this promises to open up a new method for study of biological specimens, such as muscle fibre, in which orientation exists, as well as new possibilities for crystal analysis. This arises from the relations existing between absorption and orientation of the vibrating groups whence the angle between certain characteristic bonds and the crystal axis can be determined. Measurements have already been made with minute quantities of new antibiotics and similar substances in connexion with their identification, or the diagnosis of their molecular groups.

The importance of this advance for organic chemists and biochemists cannot be exaggerated. It will surely lead to the routine study of the infra-red absorption of



many new compounds, and the relation between the spectra and constitution will therefore become still better known. As the method thus increases in power the demand for its help must also become more insistent.

I am most grateful to Dr H. W. Thompson for advance news of this remarkable development in technique, details of which will be published in a short time. Soon we shall all be repeating the wistful inquiry that ends Barrie's play: 'How much do they cost—those machines?'

I cannot pretend to be competent to discuss applied mathematics, astronomy, or physics, but it is obvious even to the uninitiated that great advances are being made in our knowledge of the mesons and of nuclear fission.

Sir George Thomson in his Bakerian Lecture gave a fascinating account of the evidence provided by study of the effects of cosmic rays on atoms, whilst Professor Lawrence with the aid of his new synchro-cyclotron has been able to produce mesons in the laboratory for the first time.

Professor E. A. Milne's forthcoming book will evoke lively discussion, for he tells me that acceptance of his theoretical system involves the assumption that Planck's constant  $h$  varies secularly with the time.

On the technical side a new type of valve which makes use of the semi-conducting properties of germanium has been introduced by Brattain & Bardeen of the Bell Telephone Co. Laboratories. This is regarded by the *cognoscenti* as an extremely significant development of electronic devices.

Coming nearer to my personal interests, the chemists and biologists of Parke Davis and Co. are to be warmly congratulated on their discovery and investigation of chloromycetin, a new antibiotic which has proved to be highly effective against scrub-typhus and certain analogous infections. The constitution of chloromycetin has not yet been disclosed, but it is said to be known, and apparently the substance has been synthesized. It makes possible the first satisfactory chemotherapy of a disease caused by a rickettsia and opens up the possibility of the control of virus diseases more generally.

Another discovery of vast medical, agricultural, and biochemical interest arises from the isolation of the intrinsic, anti-pernicious anaemia factor from liver. This brilliant consummation, a further triumph of modern chromatography, has been reached by A. Lester-Smith and his colleagues in the Glaxo Laboratories and by a team of chemists of Merck and Co. (New Jersey) led by K. Folkers. The red crystalline substance contains cobalt in a co-ordination complex which has a molecular weight of about 1600 and probably includes three atoms of phosphorus. It is proposed to call the factor vitamin B<sub>12</sub>, and it is one of the most physiologically active of known substances, a dose of 1  $\mu$ g. daily being fully effective therapeutically.

The picture is very quickly being filled in; we heard from Dr Marston quite recently of his outstanding researches on the cobalt requirements of sheep. A defect of cobalt in the soil leads to sickness and stunted growth with anaemia. Other recent and related work by Drs Tosic and Mitchell at Aberdeen concerns the assimilation of cobalt by micro-organisms. We may be sure that knowledge of the constitution of B<sub>12</sub> will suggest other correlations, perhaps with nucleotides, perhaps with folic acid, which will be of vast significance. The molecule is not especially

complex by modern standards, and its structure can be determined, provided sufficient effort is made. If I am reminded that we are unable to synthesize penicillin the reply would be that the problems are not comparable. Analysis of substances of this degree of complexity must nowadays always succeed, whereas synthesis may present a difficulty of a different order altogether. The trouble will be to get enough of the factor for investigation by ordinary methods, and this is a good illustration of the value of micro-physical devices. I am told that the infra-red absorption spectrum, determined by the new technique mentioned a few minutes ago, has already given valuable information, confirming the presence of PO, NH and OH groups, and indicating absence of aliphatic CH, but probable presence of aromatic CH as in benzene or purines.

We seem to be on the verge of great discoveries in another field perhaps not entirely unconnected with the last mentioned, that is, the problems connected with the effects of chemical substances on cell growth, including malignancy. There cannot be said to be any satisfactory treatment of cancer by chemical means, though various agents exert a beneficial effect in special cases. Such are stilboestrol, testosterone and the so-called nitrogen mustards. Of these, stilboestrol has the best claim to be regarded as a curative agent in a proportion of cases, and the halo-alkylamines ameliorate the condition of the patient. In the United States, Dr Rhoads and his colleagues have paid much attention to antagonists of folic acid. These are synthetic compounds, the molecules of which are planned to resemble those of folic acid. Several of them have been found to exert an inhibitory effect on the bone-marrow, producing anaemia, which can be reversed by folic acid. Similar antagonism is observed with bacteria, and, furthermore, Hertz has reported that certain of these substances inhibit the action of stilboestrol on the oviduct of the chick.

One of the most promising of the substances is called A-methopterin. This is folic acid with an amino-group replacing hydroxyl and a methyl group replacing hydrogen, quite a close analogue. The substance is anti-folic, anti-oestrogenic, and it has a definite inhibitory effect on the growth of tumours in laboratory animals, as well as of malignant cells in tissue culture. Folic acid is regarded as necessary for the growth of all cells, and the hope is to find a substance of low toxicity which will antagonize this factor at the level necessary to immobilize the more sensitive malignant growth without serious detriment to normal cells. It is known that laboratory work has encouraged clinical trials, but the results have not yet been disclosed.

Dr T. B. Heaton and my wife have published a preliminary account of work which finds its origin in observations made many years ago on a growth-inhibiting constituent of yeast (Heaton 1926). This was a differential growth inhibitor affecting the connective tissue cells but not the epithelia of cultures *in vitro*.

They have now prepared carbohydrate material from wheat middlings which is either itself active, or more probably contains an active principle. This produces retrogression of the implanted Walker carcinoma in rats.

It is decidedly interesting to find such a substance in a foodstuff; it may even be a vitamin. Since in some of the earlier experiments the material had been held at

a low pH, the formation of hydroxymethylfurfuraldehyde was a possibility, and it was known that many aldehydes possessed growth-inhibitory properties. It was found that hydroxymethylfurfuraldehyde, or better its polymeride, also caused the retrogression of the tumours. The connexion, if any, between these findings is not yet clear.

At the Chester Beatty Research Institute Dr A. Haddow and his colleagues have tested the effects on normal growth of a number of pyrimidine derivatives. The developments have sprung from experiments initiated for other purposes—and the sequence is of more than passing interest. It started with the yellow enzyme, and a series of experiments designed to test the effect on growth of a series of synthetic analogues of the flavins.

Administration of 9-phenyl-5:6-benzo-*iso*-alloxazine to albino rats was accidentally, and most unexpectedly, found to produce an orange-yellow pigmentation of the hair (Haddow, Elson, Roe, Rudall & Timmis 1945). Pursuing this curious observation, other coloured substances of a similar type were tested, and among them xanthopterin, the butterfly-wing pigment, first isolated by Wieland & Schöpf in 1925. This did not colour the coat but produced a significant enlargement in the size of the rat kidney. It was an actual growth of the organ due to a great outburst of cell division in the kidney tubules.

In the course of still another research, that on the carcinogenicity of many styrylpyrimidines (Haddow, Kon & Ross), it was found that two substances of this group closely allied to xanthopterin in structure had the same effect in increasing the growth of the kidney. This cannot be mere coincidence, and proves that the effect is a primary one of xanthopterin and of the related pyrimidines on the cell. The phenomenon has been observed in the rat, mouse, *Peromyscus*, the rabbit, hamster and guinea-pig.

Now xanthopterin is of natural occurrence in the kidney, and it is possible that it is present as a growth regulator. Its structural relationship with folic acid will not be overlooked. Haddow and his associates feel that it is at least as important to study the means by which normal growth is so delicately adjusted as to inquire directly why the malignant cell is unregulated.

There are further experimental foundations for these ideas which lead Haddow and his collaborators to surmise that the co-ordinated growth of the normal tissues may depend upon the supply of essential substances from an external source, and that, contrariwise, malignant cells may have acquired the power to synthesize these essential substances, or their equivalents, themselves. Such suggestions are fully consonant with all that we know of the subject as a whole and give a new prospect for the chemistry of growth and differentiation.

I am much obliged to Dr Haddow for kindly acquainting me with results and ideas which are not yet published in full and for permission to mention them to-day.

I would not like to leave this subject without an incidental reference to Berenblum's significant discovery of sensitization of tissues to the action of carcinogenic agents. The constituents of croton oil, for example, directly, or indirectly, prepare the ground for a carcinogenic hydrocarbon in a remarkable manner. The underlying

biochemical processes may be hard to bring to light, but the effort to do so would doubtless be repaid with interest.

As a drama like that of the pyrimidine group unfolds we can look back on the steps of the pioneers and be thankful that they selected the paths which led into such rich territory. But it often appeared to contemporaries that they were wandering into a barren wilderness. Whatever makes Hopkins think that there can be any possible interest in the pigments of butterflies? Why does Windaus waste his time and talent on that impossible substance cholesterol? I have heard those actual questions asked in past years, and would invite consideration of the answers that can be given to them now.

Another thought arising from recent progress is that the world of biochemistry, though of vast extent and interest, is finite, and we are beginning to sense the existence of its boundaries. We seem to encounter the same thing more often than might be expected; coincidences are indeed of frequent occurrence.

An example has already been mentioned, and another is the recent discovery that the photodynamic colouring matter of St John's wort, hypericin (Brockmann *et al.* 1939, 1942; Pace & McKinney 1941; Dhéré 1939, 1943) is closely allied to a pigment of the Aphididae, erythroaphin, studied by A. Todd and his collaborators at Cambridge (1948). And further, these substances are related to a mould pigment, oxypenicilliosin, isolated by Oxford & Raistrick (1940). The pigments are *bis-anthracene* derivatives of some kind, and it is surprising to find these little-investigated and highly characteristic substances so widely distributed in nature.

A notable coincidence in my own field of work arose very recently from a theory of Woodward regarding the course of the synthesis of strychnine in the plant. He advanced what seemed at first the fantastic idea that a benzene nucleus, originally that of dihydroxyphenylalanine (or tyrosine), suffers fission so as to give two chains which enter into further transformations.

Transferring this mechanism piecemeal into an entirely different group of alkaloids, it was found to predict that constitution of emetine which can now be experimentally demonstrated to be correct. I am glad of this opportunity to say that I was unaware until quite recently of parallel, independent work by Dr H. T. Openshaw on the constitution of emetine. This was submitted for publication some months ago and includes the establishment of one detail of the structure not fully proved by the work of Späth, of Pailer and of Karrer. A few months ago no connexion whatever could be discerned between strychnine and emetine; they seemed poles apart. Now at one stroke they are connected by recognition of a common and remarkable type of biogenesis.

# The stimulant involved in the germination of *Striga hermonthica*

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The seeds of *Striga hermonthica* will only germinate readily when they have been exposed to a chemical stimulant released from a potential host root, and the purpose of the investigation has been the examination of the chemical nature of the stimulant.

Crude concentrates have been prepared by growing roots of intact seedlings of *Sorghum vulgare* in water to which charcoal has been added, removing the charcoal and then eluting it with 70 % acetone. A residue is obtained which when redissolved in water promotes the germination of the parasite seed. It has been shown that the concentrate contains carbohydrates and particularly pentoses although aldopentoses appear to be absent. At the same time the effects on germination of a large variety of sugars has been tested and a relatively weak effect has been observed with certain samples of seed with D-fructose, D-fructose-1:6-diphosphate and with L-sorbose. The most powerful stimulation, however, has been given by D-xyloketose. This carbohydrate has given germination with all samples of seed with which it has been tested and at concentrations lower than with the crude concentrate. Maximum germination has been recorded with D-xyloketose at a concentration of  $1 \times 10^{-6}$  mg./ml. and some stimulation at  $1 \times 10^{-7}$  mg./ml.

Fresh preparations of the concentrate and of D-xyloketose are also similar in the important respects that when stored at 5 to 15° C the activities of both disappear completely within 72 hr., and that they are both completely inactivated by heating at 60° C for 60 min. Thus it is highly probable that the stimulating action of the crude concentrate is due to a substance which is either similar to or identical with D-xyloketose.

## INTRODUCTION

It has repeatedly been observed that the seeds of several species of *Striga*, like those of certain other Angiospermous parasites, when they are either in sand or soil will only germinate readily when they are in the immediate vicinity of a host root. Saunders (1933) showed however that immediate contact between the root and the seed is not required. This worker found that when the seeds are irrigated with water that has been percolated through sand in which maize is growing germination occurs in due course. This observation suggests that stimulation is due to a soluble exudate which is released by the root and absorbed by the seed. This conclusion has been fully confirmed by the results of Brown & Edwards (1944), who used a different experimental design. In this case germination was induced by applying to the seeds of *Striga lutea* water in which roots of intact seedlings of *Sorghum vulgare* had been growing for 5 days in the dark.

The present investigation is a development from these earlier observations and has been designed for the purpose of determining the nature of the material that is released from the stimulating root. The investigation has involved the elaboration of techniques for assaying the relative stimulating capacity of various solutions and for preparing concentrates of the natural stimulant. The concentrates have been

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examined chemically, and since it has been shown that they contain various carbohydrates and particularly pentose sugars the effects of several of these substances on germination have been determined. As a result it has been shown that with D-xyloketose germination may be obtained at appreciably lower concentrations than with the purest preparations of the natural stimulant so far made.

It was originally intended to study the stimulant that is involved in the germination of *S. lutea (asiatica)* and some preliminary observations were made with this species. Unfortunately we were unable to obtain sufficient quantities of the seeds and the investigation has had to be continued with seed of *S. hermonthica*. All the results given below (with one exception) refer to tests made with *S. hermonthica*.

#### ASSAY TECHNIQUE

The technique depends on the condition described by Brown & Edwards (1946), that the proportion of seeds that germinate within a limited time decreases with increasing dilution of a given stimulating solution.

In the normal procedure the stimulating capacity (referred to below as the activity) of a particular solution is assessed as follows: A series of dilutions is prepared each being a tenth of the concentration of the preceding one. With each dilution six separate tests are made, in each of which a group of parasite seeds is suspended in a drop of the solution. After incubation for 24 hr. at 35° C the percentage germination given by each test is determined and a mean figure calculated from the six individual percentages. A small proportion of seeds always germinates in water and since it is occasionally necessary to determine the highest dilution at which stimulation is observed, in addition to the tests made with the experimental solution six replicate tests with water only are included in the assay. A further elaboration is required by the condition that the percentage germination given by a solution of constant activity is not always the same even with the same sample of seed. Thus a standard must be provided against which the germinations given by the experimental solution may be compared, and in the assay in addition to the other tests six replicates with a solution of standard activity are included, the mean values given by the experimental solutions being compared with the mean value derived from the standard solution.

A representative series of results for a single assay is given in table 1.

From data such as those of table 1 the lowest concentration is estimated which gives half the percentage germination given by the standard and this concentration, expressed as the several fold dilution of the experimental solution is taken to represent the activity of that solution. The activity is determined by plotting percentage germination against the logarithm of the several fold dilution over the appropriate concentration range. Since the lowest concentration is required which gives the half value, the appropriate range is that which extends from the concentration which gives maximum germination to the lowest giving a greater percentage germination than water. An arbitrary straight line is drawn through the plotted values, and the dilution corresponding to the half value is read off from this curve. The value so determined is logarithmic and requires conversion to standard units.

The application of this method to the data of table 1 gives an activity of 3000 for the solution E. Half the percentage germination given by the standard is taken as the reference value, since the maximum given by the experimental solutions may be higher or lower than that of the standard, and the rate varies at which germination increases or decreases with increasing dilution. In certain cases a ten-fold dilution of a solution which gives a maximum value is sufficient to extinguish stimulation, in other cases some stimulation may still be observed after a thousand-fold dilution. Values for activity are given to the nearest million, thousand, hundred, ten or integer when they are greater than one million, one thousand, one hundred, ten and one respectively.

TABLE 1. RESULTS (PERCENTAGE GERMINATIONS) FOR A SINGLE ASSAY

(S represents 'standard solution', W water, E the undiluted experimental solution and values in first column the fractional concentrations of this solution)

	results with replicate tests						means
S	46.4	41.6	38.9	48.5	43.7	44.7	44.0
W	2.0	3.0	2.2	3.9	0.0	4.2	2.6
E	2.4	1.9	5.9	0.0	6.1	2.0	3.1
$1 \times 10^{-1}$	4.8	14.6	6.1	5.0	3.8	4.9	6.5
$1 \times 10^{-2}$	6.4	2.6	2.1	6.7	3.3	2.3	3.9
$1 \times 10^{-3}$	38.3	33.8	32.0	40.5	34.3	25.0	34.0
$1 \times 10^{-4}$	9.4	3.6	5.3	2.8	3.8	3.3	4.7
$1 \times 10^{-5}$	2.4	2.9	2.6	0.0	4.4	0.0	2.1

All the solutions whose activities are assayed are prepared from dry materials dissolved in glass distilled water at a concentration of 1 mg./ml., and the undiluted solution E of table 1 has this concentration. Therefore each fractional concentration of the assay represents the same fraction of one mg./ml. In table 1 the highest observed germination with the experimental substance is given at an absolute concentration of  $1 \times 10^{-3}$  mg./ml., and some germination (referred to below as the significant germination) is given at  $1 \times 10^{-4}$  mg./ml. (1:10,000,000).

In the general assay procedure three sets of techniques are involved which may be considered separately. The three techniques are those required in (a) determining percentage germination, (b) preparing the standard solution, and (c) preparing the parasite seed for the assay.

#### *Determination of percentage germination*

The seeds are small being only  $0.4 \times 0.2$  mm., and the cultures in which germination is determined must therefore be such that they can be examined microscopically. This necessarily limits the volume of fluid that can be used in each test culture. At the same time the fluid drop must be protected against evaporation since it is necessary to maintain the liquid volume constant, and further the seeds must be adequately aerated during incubation. These conditions have been met by an adaptation of the normal hanging drop technique. The parasite seeds are dispersed in 0.01 ml. of experimental fluid applied to the lower surface of a cover-slip which is sealed at the edges to a metal or glass ring, the lower edge of which is

in turn sealed to a microscope slide. The whole culture is incubated at 35° C for 24 hr. and at the end of this time the total number of seeds in the drop and the number that have germinated are counted and from these two figures the percentage germination is calculated. This technique has been described elsewhere in detail by Brown & Edwards (1944).

The mean figure for the group of six replicate cultures is determined from the percentage values and not from the absolute numbers that germinate in each culture, since the total number varies considerably. This variation is a consequence of the method that has to be used for transferring the seeds to the drop from a dish in which they have been pretreated (see below). This operation must be performed more rapidly than is possible if they are counted on the stage of a microscope, and they are therefore carried on the point of a needle as a cluster which is adjusted by eye. The number of seeds in the cluster varies between 20 and 100 but Brown & Edwards have shown that this variation with the size of drop used does not affect percentage germination.

#### *Preparation of standard solution*

As shown below the stimulant is highly unstable and the activity of even a dry preparation of active material decreases rapidly with time. Thus a solution with constant activity cannot be prepared from a stock of dry concentrate. Brown & Edwards (1944) showed however that a solution with a constant activity is obtained by allowing the roots of uniform host seedlings of *Sorghum vulgare* to develop in distilled water in the dark for a standard period of 5 days at a constant temperature of 25° C. Twenty seedlings are selected from a petri dish culture in which seeds of *Sorghum* have been germinated, they are transferred to a float which consists of a length of thin glass tubing closed at both ends and bent into the form of a narrow U. The float rests on the surface of 20 ml. of glass-distilled water in a boiling tube which is slanted at an angle of about 20° with the horizontal. When the float is in position the tube is closed with a rubber stopper. The seedlings develop rapidly with the roots growing down into the water between the two arms of the float. After incubation for 5 days the water is withdrawn and used immediately as the standard solution. The stimulation that is given by this solution decreases rapidly with time, and a fresh preparation must be used for each assay.

#### *Preparation of parasite seeds*

If initially dry parasite seeds are placed on filter paper and moistened at intervals with water that has been in contact with host roots some germination occurs after a period that varies between 3 and 7 days when the culture is incubated at 35° C. This general arrangement, however, cannot be used as the basis for an assay since solutions of the stimulant rarely retain any activity after storage at room temperature for 48 hr. On the other hand germination may occur within 24 hr. after application of the stimulating solution if the seed has been incubated in contact with water at 25° C for some days previously. During the incubation at 25° C no germination occurs but as the period of incubation is extended the germination given after application of a standard stimulant solution increases



until a maximum value is reached and then declines with further extension of the period of incubation. This effect which has been called the 'pre-treatment effect' is shown by the data of table 2 which were obtained with two independent treatments from one of the samples of *Striga hermonthica* used in this investigation. The agreement between the two sets of determinations is close and it shows that the potential percentage germination (referred to below as the germination capacity) that is given with the standard solution can be closely controlled when the same sample of seed is being used.

TABLE 2. DUPLICATE DETERMINATIONS OF CHANGE IN GERMINATION CAPACITY WITH TIME OF PRETREATMENT AT 25° C

time (days)	mean percentage germination	
	I	II
4	12.9	7.7
8	29.1	32.3
12	31.2	33.0
16	15.7	16.5
20	2.9	2.7

Since the prior treatment of the seed in the manner indicated provides the conditions for rapid germination after application of the stimulant this treatment forms part of the assay procedure. The seeds are placed on sintered glass disks which are accommodated in small petri dishes containing enough water to saturate the disk. The cultures are incubated at 25° C in the dark for an appropriate period, samples being taken from each dish at intervals of not less than 3 days. The seeds are light sensitive (Edwards 1946) and exposure to light during the withdrawal of samples at intervals of less than 3 days markedly depresses germination capacity. Although germination capacity is changing with time and affects the germination given with an experimental solution, this is compensated by the corresponding effect given with the standard solution. The variation in germination capacity, however, affects the accuracy of the assay to the extent that it influences the sampling error. When germination capacity is high the standard deviation within six replicates is small but when it is low the deviation is of course relatively greater. This source of error could be avoided by prior incubation for the standard time necessary to bring the seed to the highest germination capacity. The difficulties of treating a quantity of seed sufficient for a single assay are however considerable and would involve an elaboration of the technique that would restrict the scope of the enquiry.

#### *Accuracy of the assay*

Assays of this type should of course be made with a standardized biological material. Unfortunately the seed available for this investigation had been collected in different parts of the tropics, and the samples varied with regard to degree of maturity and age since harvesting. The maximum germination given with different samples has varied from 10 to 60 %, and the different samples have therefore involved from 90 to 40 % of grains which did not germinate even in the most

favourable circumstances. Some of the seeds in all samples are undoubtedly sterile and non-viable; many of those that do not germinate, however, are probably immature grains in which the process of after ripening is not complete. It has been observed with some samples that the maximum number that germinates increases with age, and the rate at which the maximum is reached in pretreatment also tends to increase.

With seed of low germination capacity the accuracy of the assay is of course correspondingly low. But with seed giving a percentage germination greater than 10 % with the standard, when duplicate assays are made with the same seed the results do not differ by more than 100 %. With different samples of seed however the percentage difference may be greater. The following pairs of activity values are the results of duplicate determinations made with different samples of seeds: 20 and 100, 10,000 and 18,000, and 2,000,000 and 3,000,000 (the last pair of results were obtained with *S. lutea*). It may be emphasized that although the percentage difference between replicates is large the range of values over which activities are expressed extends from one to ten million, and for all values of less than a million the differences are only very small percentages of the total range.

#### PREPARATION OF CRUDE CONCENTRATES

It was found that active solutions prepared by suspending the roots of intact plants in water were inactivated when shaken with charcoal. For instance a solution that gave 43.3 % germination gave only 5.5 % after treatment with animal charcoal at the rate of 150 mg. to 200 ml. of fluid. It was also found that an acetone eluate of the charcoal yielded a residue which when re-dissolved in water induced the germination of the parasite seed. The data given in table 1 were obtained with a typical concentrate obtained in this manner.

In the preliminary stage of the investigation concentrates were prepared by adding charcoal to active solutions after they had been withdrawn from the cultures. It was found, however, that the yield of the crude concentrate could be increased by adding the charcoal to the water at the time the cultures are set up. The reason for this enhanced yield has not been determined but it is probably an indirect effect of a more vigorous growth of the seedlings. It has been observed repeatedly that roots in water to which charcoal has been added grow more vigorously than they do when charcoal is not provided. Brown & Edwards (1944) found that the stimulant is exuded from the root meristem and it is therefore probable that the greater yield is due to a greater meristematic activity.

These preliminary observations formed the basis for the technique finally developed for preparing crude concentrates. In the standard procedure seedlings are cultured in pneumatic troughs 12 in. in diameter and 6 in. deep. Eighteen grams of charcoal and 1.5 l. of distilled water are placed in each trough. A float consisting of muslin stretched across a closed glass ring carries the seedlings on the surface of the water.

Considerable difficulty has been experienced in obtaining a suitable grade of charcoal, since it must not affect the pH of the solution, and must contain negligible

amounts of impurities. Sutcliffe and Speakman 'Genster' charcoal has finally been adopted as the most suitable material for the present purpose.

Seedlings of *Sorghum vulgare* have been used throughout; they are developed from seeds on damp filter paper at 25° C and when the roots are about 1 cm. long and the coleoptiles about half this length, after removal of the ungerminated seeds, the seedlings are scattered over the surface of the floats, about 1000 being allocated to each trough. The muslin of the float is of course saturated and dry seeds placed on it do not germinate, but when some growth has occurred the seedlings develop vigorously with the roots growing through the muslin and into the water.

Thirty to forty cultures are set up each week. They are placed for 5 days in a chamber in near-darkness in which the temperature is maintained at 25° C. At the end of the 5-day period the charcoal is separated from the water by filtration.

The proportion of water to charcoal in each dish is that which in a preliminary series of experiments was found to give the highest yields of crude concentrate. If the quantity of charcoal is reduced the yield from each dish decreases and if the volume of water is reduced the growth of the seedling is affected. Although some charcoal in the culture fluid promotes the growth of the roots quantities greater than those normally used inhibit growth.

With this technique 5 to 10 mg. of concentrate are obtained from each dish and the total weekly production is about 200 mg.

#### COMPOSITION AND PROPERTIES OF THE CRUDE CONCENTRATE

The stimulant is recovered from charcoal by elution with 70 % acetone. With this treatment 80 % of the concentrate is removed in the first extraction.

The activities of different samples of the concentrate have varied considerably. Values ranging from 50 to 100,000 have been recorded. At various stages the sample of host seed has been changed and so has the type of charcoal and it has been found that the productivity of the different strains of *Sorghum* varies and also that the amount and type of impurity in different samples of charcoal varies considerably.

The data of table 1 show that the highest concentration of 1 mg./ml. at which these preparations are applied to the seed gives little or no stimulation. With increasing dilution percentage germination increases until a peak value is reached after which further dilution is accompanied by decreasing germination. The peak values are of the same order as those given with the standard solution and in some cases they are higher. Clearly with the optimum concentration germination approaches the full capacity of the seed and the absolute concentrations at the optimum are therefore of some significance. The lowest optimum concentration recorded is  $1 \times 10^{-4}$  mg./ml., values of the order of  $1 \times 10^{-3}$  mg./ml. are the most commonly observed. It may be emphasized that these concentrations represent one part in ten million and one part in one million of water. Clearly if the full capacity of the seed is realized with concentrations of this order the proportion of the stimulant in the crude concentrate is probably large. This conclusion is also indicated by the lowest concentrations with which significant germinations are observed. Stimulation has occasionally been recorded at concentrations of  $1 \times 10^{-6}$  mg./ml. and frequently at concentrations of  $1 \times 10^{-5}$  mg./ml.

The concentrate separated from the charcoal varies in appearance from a colourless glass to a white hygroscopic amorphous solid according to the inorganic content which varies between 15 and 40 % (estimated by ashing).

The chemical purification of the stimulant is a matter of considerable difficulty since the activity of the concentrate decreases very rapidly with time when stored at room temperature, and normally disappears 72 hr. after isolation from the charcoal. Nevertheless a further concentration of the organic material has been achieved by methanol extraction which gives a methanol-soluble syrup and a solid methanol-insoluble fraction. Most of the activity is retained in the soluble fraction. A representative determination gave activity values for the soluble and insoluble fractions of 1000 and 1 respectively. The residual activity of the insoluble fraction is presumably due to adsorption.

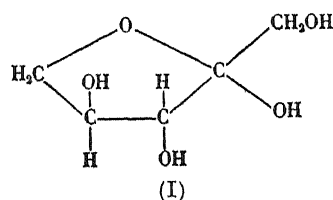
Removal of the last traces of inorganic salts from the methanol-soluble syrup by the use of the ion-exchange resins Zeocarb 215 and Deacidite have invariably led to complete deactivation of the concentrate. All chemical operations on the concentrate have been conducted in an atmosphere of nitrogen.

The methanol-soluble fraction gives a strong positive Molisch reaction for carbohydrates, is optically active (one sample gave  $[\alpha]_D^{20} = +20^\circ$ ) and readily reduces Fehling's solution on gentle warming. Preliminary evidence as to the nature of the carbohydrates has been sought from various colour reactions applicable in the series (Tollens-Elsner 1935). It has been found that a positive pentose reaction is obtained with orcinol and hydrochloric acid (modified method of Militzer 1946) although the characteristic aldopentose colour reaction with  $\beta$ -naphthol and sulphuric acid has not been observed. A weak keto-hexose reaction with diphenylamine and hydrochloric acid or with resorcinol and hydrochloric acid (Seliwanoff) has been obtained, although no 2-deoxy sugars (Keller-Killiani) or alduronic acids (naphthoresorcinol) could be detected. Periodate oxidation does not yield any acetaldehyde, indicating the absence of methyl sugars (Nicolet & Shinn 1941). On reaction with *o*-nitrophenylhydrazine an *o*-nitrophenylosazone is obtained which is identical with that derived from glucose, mannose or fructose. The characterization of this sugar component has not been attempted at this stage of the investigation since further work (see below) has shown that the presence of these monosaccharides would only account for a very small portion of the total activity of the natural concentrate.

Further work on the concentration and identification of the active components of the crude concentrate is in progress and will be reported at a later stage, but meanwhile since the chemical examination of the methanol-soluble fraction has shown that various sugars and particularly pentoses are present, the effects of a wide range of sugars and sugar derivatives on the germination of *Striga* seeds have been examined and some have been found to stimulate germination. A large number of substances other than sugars have also been tested but none of these has given any stimulation within the same concentration range and time that it is given by the natural stimulant. Brown & Edwards (1945) reported that germination was induced in seeds of *Striga lutea* by treatment with thiourca and allylthiourca. This observation has been confirmed with *S. hermonthica* but it has also

been confirmed that the seeds must be treated for at least 3 days with high concentrations of these compounds (1 to 10 mg./ml.).

The effects of 30 sugars and allied substances have been examined and the following have been found to be completely inactive: L-xylose, D-arabinose, L-arabinose, D-ribose, D-lyxose, D-mannose, L-erythrulose, D-adonose, L-xyloketose, D-tagatose, L-psicose, D-psicose, sucrose, cellobiose,  $\alpha$ -D-glucose-1-phosphate,  $\beta$ -D-glucose-1-phosphate, D-glucose-6-phosphate. The following, with low activities of less than 500, have given some stimulation: D-xylose, L-rhamnose, D-glucose, D-galactose, L-adonose, maltose, lactose, ascorbic acid, and galacturonic acid. With certain samples of seed D-fructose, L-sorbose, and D-fructose-1:6-diphosphate have given activities greater than 500. The highest activities, however, have always been given with D-xyloketose; moreover this sugar has promoted germination in all samples of seed with which it has been tested. Also all samples of D-xyloketose (I) that have been prepared have at some time given some activity, although for reasons that are discussed below, while certain preparations from the same sample may give high activity, others may give negligible activity.



Four representative sets of results with different samples of D-xyloketose are given in table 3.

TABLE 3. MEAN PERCENTAGE GERMINATION AND ACTIVITIES FROM  
FOUR SAMPLES OF D-XYLOKETOSE

	(Symbols as in table 1)			
	I	II	III	IV
S	30.5	37.9	15.6	34.8
W	4.9	2.6	0.8	5.1
E	2.3	2.3	39.8	16.0
$1 \times 10^{-1}$	3.3	2.9	22.5	31.6
$1 \times 10^{-2}$	19.1	12.1	13.6	21.7
$1 \times 10^{-3}$	30.3	6.6	1.9	19.9
$1 \times 10^{-4}$	9.0	18.3	1.7	17.6
$1 \times 10^{-5}$	10.4	21.6	0.3	13.4
$1 \times 10^{-6}$	0.0	23.0	0.3	6.2
Activity	12,000	>1,000,000	300	8,000

With some samples of D-xyloketose the activity is greater than with the crude concentrate; maximum germination and significant stimulation having been recorded at concentrations of  $1 \times 10^{-6}$  and  $1 \times 10^{-7}$  mg./ml. respectively. It may be noted in this connexion that even with samples in which the activity is low the stimulation is clearly not due to a nutritive effect. With Sample III of table 3 for instance some stimulation is given at a concentration of  $1 \times 10^{-2}$  mg./ml. which

is one part per hundred thousand of water. Clearly at this concentration any effect due to the metabolic breakdown of the sugar is highly improbable.

Further, although the stimulation given with D-xyloketose as with the other sugars varies considerably the stimulation is probably not due to impurities in the samples of sugar used for the following reason. The D-xyloketose used in this investigation has been prepared by two unrelated methods, and this sugar is always active when freshly prepared. In the first place D-arabitol has been submitted to bacterial oxidation by *Acetobacter suboxydans* and the crude product purified through the crystalline 2:3-monoacetone compound (m.p. 68 to 69° C) (Prince & Reichstein 1937) and regenerated by hydrolysis with aqueous oxalic acid (Levene & Tipson 1936). The regenerated sugar was a colourless syrup  $[\alpha]_D^{20} = -32^\circ$  (Schmidt & Treiber 1933 give  $[\alpha]_D^{18} = -33.2^\circ$ ). Secondly, D-xylose has been rearranged by heating with dry pyridine under reflux and the crude sugar purified through the crystalline *p*-bromophenylhydrazone, m.p. 128 to 129° C (Schmidt & Treiber 1933 give m.p. 128 to 129° C) and regenerated by warming with an aqueous solution of benzaldehyde.

As indicated above the activities given by D-xyloketose and other sugars vary widely. The conditions that determine this variation are undoubtedly complex, but one of them is probably variation in the condition of the seed. Different strains of organisms differ with regard to their requirements for growth factors, and some evidence is available which suggests that seeds of *Striga hermonthica* from different geographical areas differ with regard to their stimulant requirements. Certainly seeds of *S. lutea* germinate with lower concentrations of the stimulant than do those of *S. hermonthica*. At the same time it is also probable that different strains and samples of different ages differ with respect to the capacity to substitute other sugars for the natural stimulant and this probably explains the variability of the response to such sugars as L-sorbose and D-fructose.

#### COMPARISON OF D-XYLOKETOSE AND THE NATURAL STIMULANT

Preparations of D-xyloketose and of the crude concentrate are similar in one important respect. Observations have been made at intervals of 24 hr. on samples of each stored at 5 to 15° C, and it has been found that within 48 to 72 hr. after receipt of the material at Leeds from Cambridge the activity has disappeared. This occurs with both, whether they are in the dry state or in solution. Further, the activities of both the concentrate and the sugar are affected similarly by higher temperatures. Solutions of both are completely inactivated by heating at 60° C for 60 min.

The activities of solutions of D-xyloketose and of the concentrates are, however, differentially affected by changes in hydrogen ion concentration. With the concentrate the highest activity is given at pH 4 and the activity tends to decrease as the pH is raised to 7, with D-xyloketose on the other hand the activity is less at pH 4 than it is at pH 5 but about the same between pH 5 and 7. Further differences between the concentrate and D-xyloketose have been noted in connexion with the effect of dilution on percentage germination. With the concentrate, after a peak

value is reached, percentage germination tends to decrease sharply (as in table 1); with the sugar on the other hand it does so over a greater range of decreasing concentration (as in table 3). When the percentage germination is low and the error correspondingly high, the effect of the slow decrease may be to give apparent secondary peaks in the curve relating percentage germination to concentration.

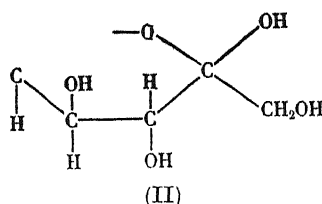
In this connexion it may be noted that the wide variation in activities recorded with the sugar and with the concentrates must be attributed primarily to the high instability of the preparations. Different periods of 1 to 3 days have elapsed in this investigation, between the time of preparation of the samples of D-xyloketose or of the concentrate, and that of the first biological test in each series. The variable delay has been due to the time required for postal transmission of the samples, sent as aqueous solutions sealed under nitrogen, from Cambridge to Leeds.

### DISCUSSION

Clearly since the chemical examination of the crude extract indicates that it contains considerable quantity of pentose sugars which are not aldopentoses, and since D-xyloketose may stimulate germination at lower concentrations than the crude concentrate, it is highly probable that the activity of the concentrate is due to a substance which is either very similar to or identical with D-xyloketose. This conclusion is further justified by the observations that activity with both the sugar and the concentrates in solution and in the dry state decreases similarly with time. Although the activity of the concentrate is probably due to a substance similar to D-xyloketose, differences between the stimulating properties of the sugar and the concentrate might be expected, since the latter certainly contains many other substances in addition to the sugar, and the differences noted above are no doubt a result of this factor.

The nature of the changes involved in the deactivation of both D-xyloketose and the natural stimulant have not been determined, but the possibility is now being examined that the change in activity is not due to a comprehensive change of all the carbohydrate or a derivative but to a limited change that leads to the formation of small amounts of an inhibitor.

Although it is not possible to make any comprehensive generalization relating chemical structure and biological activity on the limited number of substances examined, it is significant that all of the ketoses which have been found to be active have the hydroxyl groups in the *trans* position on the 3- and 4-carbon atoms i.e. as in (II), whereas the *cis* analogues have proved to be invariably inactive.



Further, the stereochemical configuration of the sugar is of primary importance as L-xyloketose has been shown to be devoid of biological activity.

We wish to acknowledge our indebtedness to Professor T. Reichstein for samples of several sugars and sugar derivatives and to Dr R. Kline and Dr F. Bergel of Roche Products Ltd., for assistance with the bacterial oxidation of D-arabitol. Our thanks are also due to Dr J. C. May of the Empire Cotton Growing Corporation, to Dr J. E. Peat of the Agricultural Department, Tanganyika for generous gifts of *Striga* and *Sorghum* seeds, and to the Colonial Products Research Council for its support of the investigation.

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## Metabolic changes which form the basis of a microbiological assay of nicotinic acid

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The manner in which limitation of nicotinic acid in cultures of *Lactobacillus arabinosus* limited their formation of titratable acid has been examined. This system forms the basis of a much-used microbiological assay of nicotinic acid.

During a large part of a normal assay, the rate of formation of titratable acid was proportional to the quantity of added nicotinic acid. The relationship between this rate and the quantities of nicotinic acid has been expressed as a ratio: mol. titrated acid/hr./mol. bacterial nicotinic acid, which has been called the *catalytic activity* of nicotinic acid in the assay system. Its value was about  $15 \times 10^3$  hr.<sup>-1</sup> at pH 6.8, and fell with fall in pH to  $5 \times 10^3$  hr.<sup>-1</sup> at pH 5.0.

The rate of acid formation during assay fell to a greater extent than could be accounted for by the change in pH which took place during the assay. Much of this fall was due to loss of nicotinic acid from the bacterial cells. During the first day's growth the added, limiting, quantities of nicotinic acid were rapidly and completely removed from the culture fluid to the cells, but following this period the nicotinic acid was gradually lost. Loss of nicotinic acid from the cells bore a simple relationship to the quantity of titratable acid formed; for each



$5 \times 10^5$  mol. of acid produced, 1 mol. of nicotinic acid was lost. This molar ratio has been called the *catalytic capacity* of nicotinic acid for the formation of titratable acid in the present system. Its value was independent of the changes in pH during assay and of the actual rate of acid formation.

The use of the terms catalytic capacity and catalytic activity has been assessed, and relationships between them and the quantities of titratable acid produced at different times have been expressed mathematically. During the assay as it is ordinarily performed, only part of the catalytic capacity of the added nicotinic acid was found to be used. About half the acid remained in the cells, and these remained capable of the formation of titratable acid, after the 3 days at which the acid formed in assay cultures is commonly titrated.

Because nicotinic acid was lost from the bacteria in proportion to their formation of titratable acid, the assay system could compensate for initial fluctuations in such circumstances as temperature or the quantity of bacterial cells.

## INTRODUCTION

Microbiological assays, now widely used for estimating vitamin-like compounds, have been developed largely empirically. They however exhibit regularities which are shown below to give information on the metabolic changes undergone by vitamins in living organisms.

Thus an assay of nicotinic acid by *Lactobacillus arabinosus* is carried out by titrating the quantities of lactic acid formed in cultures containing glucose and varying quantities of nicotinic acid. It was found that limitation of nicotinic acid limited: (1) the quantity of organisms formed; (2) the rate of acid formation; and (3) the total quantity of acid produced when that ceased to increase rapidly. It was found also (4) that measurement of the acid produced gave more reliable results than did the measurement of microbial growth, and that no direct proportionality existed between the quantity of organisms formed and either the rate of acid production or its final amount. On the basis of a common supposition that nicotinic acid affected acid production by limiting growth, results (1) and (2) would be anticipated but not results (3) and (4). This assay has been examined and reciprocal connexions found between the organism's metabolism of glucose and of nicotinamide.

## EXPERIMENTAL

The method investigated was that employing *L. arabinosus* 17/5 and developed by Snell & Wright (1941), Krehl, Strong & Elvehjem (1943) and Barton-Wright (1946). The medium and general conditions were exactly those of Barton-Wright (1946) except where otherwise stated. We employed as acid-hydrolyzed casein (at a concentration in the final medium equivalent to 1 % of casein) the preparation of McIlwain & Hughes (1944). The inoculum was grown by subculture from an agar slope to 5 ml. of assay medium containing  $1\mu\text{g.}$  of nicotinic acid. Incubation throughout was at  $37^\circ\text{C.}$  in most cases in an ordinary bacteriological incubator. In those experiments in which the early part of growth curves was followed with precision, cultures were maintained at  $37^\circ \pm 0.05^\circ\text{C.}$  in a thermostatically controlled tank and shaken with a double oscillation of 8 cm. each sec. Acid production in 1 or 2 ml. aliquots was measured by titration with 0.1 or 0.05 N-NaOH. Bacterial growth was determined by measuring the optical densities of 0.5 ml. specimens of cultures (or of known dilutions of cultures) with a Hilger photoelectric absorptio-

meter, using neutral filters and micro-cell attachments. Optical densities ( $O$ ) were compared with bacterial weight ( $w$ ; mg./ml. after washing with 0.9% NaCl and drying at 100°) in several batches, and the relationship  $O \times 0.34 = w$  was found. Bacterial weights quoted throughout are dry weights.

To prepare materials for assay, solutions or suspensions were made normal with respect to HCl, autoclaved at 15 lb./sq.in. for 20 min., made to pH 6.8 by NaOH and to a standard volume, and centrifuged free from any suspended matter. For experiments with non-proliferating suspensions, organisms were grown in quantities of 100 or 300 ml. of the assay medium, prepared with varying quantities of nicotinic acid. After growth the cells were collected by centrifuging, washed and resuspended in 0.9% NaCl.

## RESULTS

### (1) *Relations between acid formation and growth during assay*

#### (a) *Typical assay curves (relating response to dosage)*

By the present method, response of the organisms to nicotinic acid is normally measured by titrating the quantity of acid which has been formed during 3 days' incubation. Figure 1 shows that with quantities of nicotinic acid between 0.2 and 1.2  $\mu\text{mol.}/10\text{ ml.}$  culture, this response is regular and bears an almost linear relationship to the quantity of added nicotinic acid. Such a response has been one of the aims of the investigators who devised the present assay conditions; we wish to inquire how this result has been achieved.

The other curves of figure 1 show that this acid has been formed gradually, and that the quantity of titratable acid formed by the first day is not so simply related to the quantity of added nicotinic acid. Increasing quantities of nicotinic acid within this range produce also increasing quantities of bacteria (figure 2), but the relationship between added nicotinic acid and quantity of bacterial growth is much less regular than that between nicotinic acid and acid titre. In the assay system some features therefore exist which ensure that a given amount of nicotinic acid will lead to the formation of a given amount of titratable acid from glucose, although the glucose is always present in excess and the quantity of organisms which produce the acid may vary.

#### (b) *Course of formation of acid and bacteria; their relative independence*

Figures 3 and 4 show the course in time of bacterial growth and the formation of acid. They show that during the formation of a large part of the acid of an assay tube, the bacteria are growing to a very small extent only. The independence of acid formation and growth was shown even more clearly in the following experiment. A culture deficient in nicotinic acid was grown until its mass of growth and quantity of acid had reached approximately stable values. At this point (5 days; figure 5) a further quantity of nicotinic acid, equal to that present initially, was added. This resulted in the prompt formation of further acid, which again approached a limit after a few days. Its final quantity was about twice that given by the first dose of nicotinic acid. It was found, however, that the quantity of cells present in the

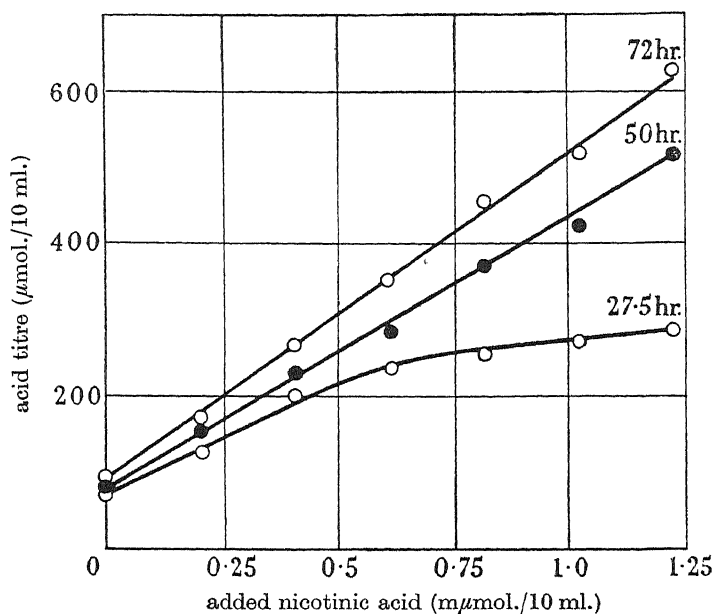


FIGURE 1. Standard assay curves, relating the quantity of acid formed at different times in cultures of *L. arabinosus* to the quantity of added nicotinic acid.

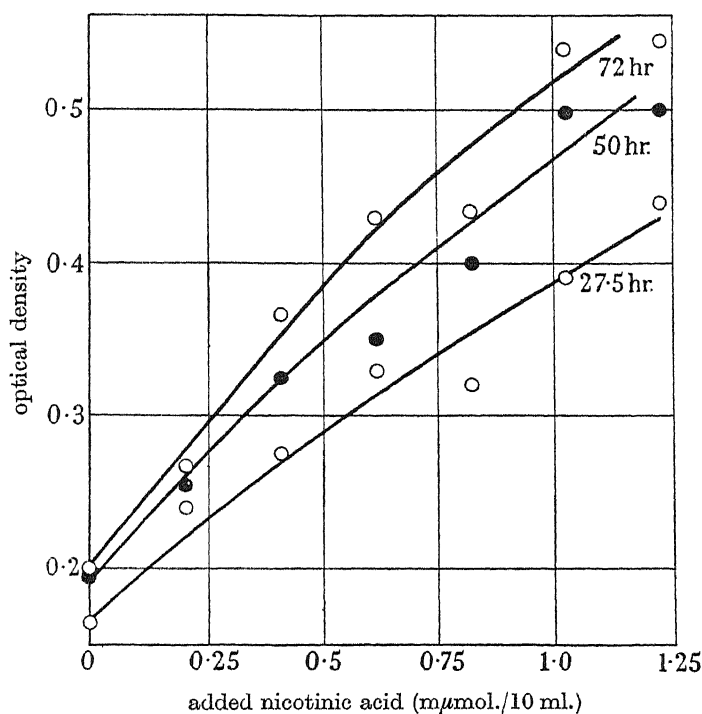


FIGURE 2. Mass of growth produced in the cultures of figure 1, and measured by their optical densities.

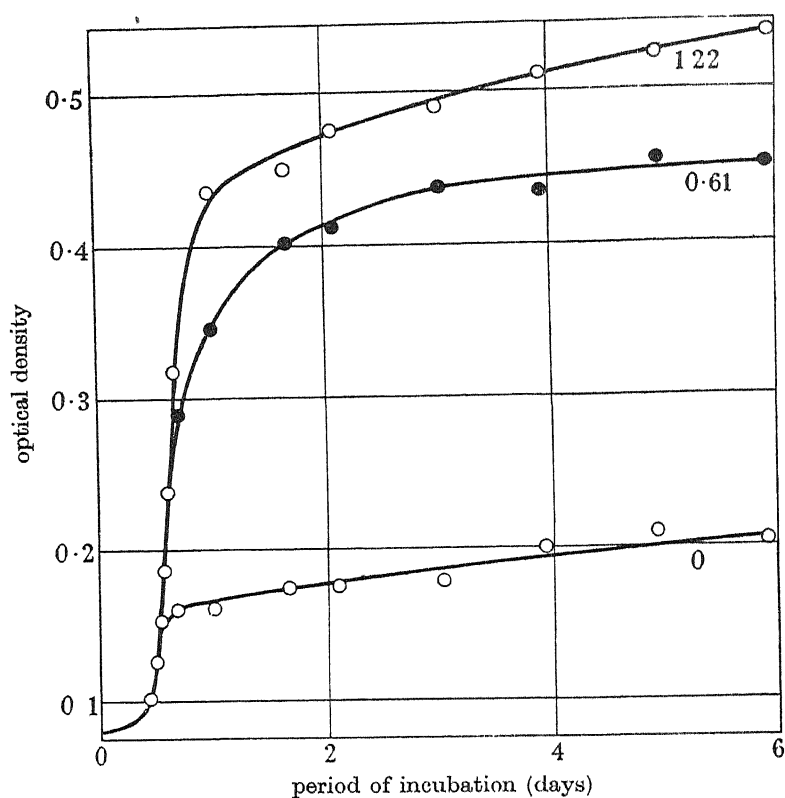


FIGURE 3. The course of growth of cultures of *L. arabinosus*, measured by their optical densities. Different lines refer to different cultures, containing initially the quantities of nicotinic acid which are indicated (in  $\mu\text{mol./10 ml.}$ ).

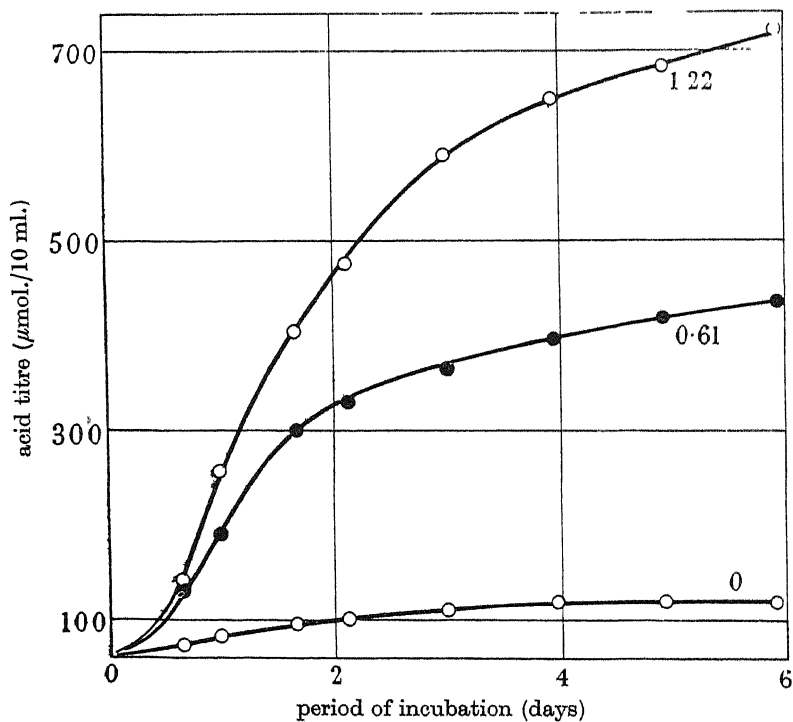


FIGURE 4. The course of acid formation in the cultures of *L. arabinosus* referred to in figure 3.

culture was almost unaffected by the second addition of nicotinic acid (figure 5.) Subcultures showed the cells still to be viable. Here, the process of acid formation had been completely dissociated from growth. A possible interpretation of the limited acid formation in earlier experiments was that a given quantity of acid produced a given quantity of cells, which slowly died or became generally enfeebled. Decay is now seen to be confined to systems specifically related to nicotinic acid, as addition of nicotinic acid only, caused resumption of acid formation.

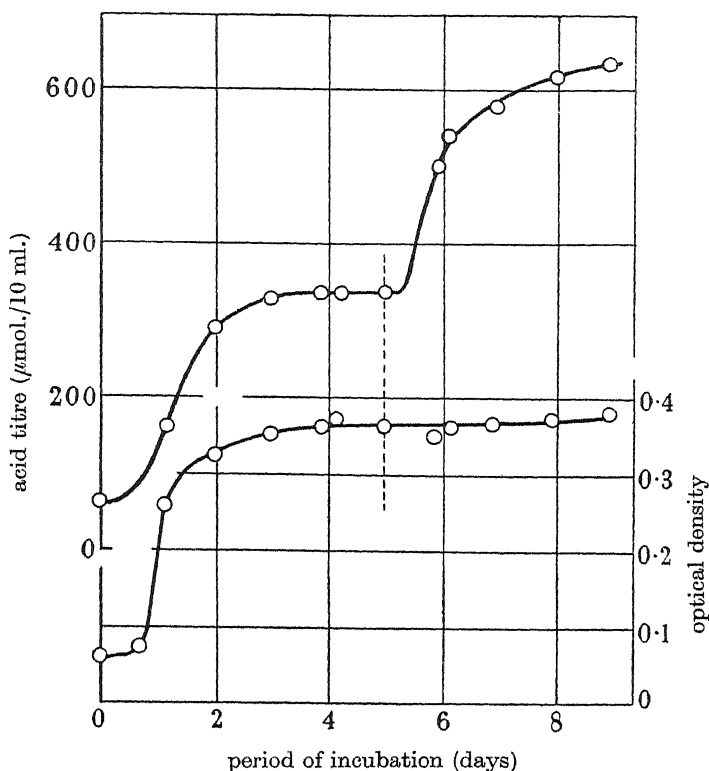


FIGURE 5. The effect of an addition of nicotinic acid ( $0.61 \mu\text{mol.}/10 \text{ ml.}$ , at the point indicated by the dotted line) to a culture of *L. arabinosus*, 5 days after inoculation. Initial nicotinic acid: also  $0.61 \mu\text{mol.}/10 \text{ ml.}$ . Below: quantity of bacteria, measured by optical density. Above: quantity of acid formed.

(c) *Rate of acid formation; the catalytic activity of nicotinic acid*

The course of acid formation has therefore been studied in more detail. *L. arabinosus* was grown in triplicate series of flasks containing 50 ml. of assay medium with varying quantities of nicotinic acid. These were sampled at intervals and the titratable acid determined. Results are given in figure 6. It will be seen that the curves at each time, when extrapolated to intersect the nicotinic acid-axis, do so at nearly the same point. This is interpreted as representing nicotinic acid in the medium before additions were made. In the present experiment the quantities concerned were  $0.23 \mu\text{mol.}/10 \text{ ml.}$ , and in subsequent calculations this has been included in the content of nicotinic acid initially present in each flask. A series of

acid-formation/time curves similar to that of figure 4 was constructed from the data of this experiment, and the slopes of the curves at different times determined graphically. Values were thus obtained for the rate of acid formation, and in figure 7 these rates are plotted against the total quantity of nicotinic acid initially present in the cultures. The curves of figure 7 show the characteristics of the following two paragraphs.

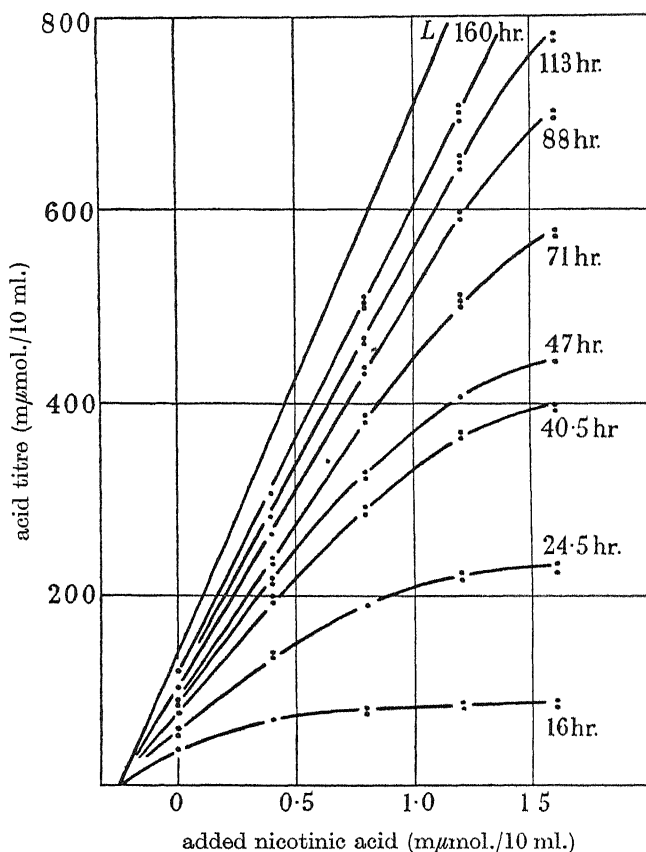


FIGURE 6. The titratable acid produced at different times after inoculation (given on the figure, in hr.) from limited nicotinic acid, by *L. arabinosus*. Each determination was in triplicate. Line L, limiting value derived theoretically; extrapolation: see text.

(i) *Apparent catalytic activity*. With smaller quantities of nicotinic acid, the rate of acid formation increased linearly with increase in nicotinic acid. At 18 hr., 1 mμmol. of nicotinic acid could lead to the formation of some 12,000 mμmol. of titratable acid/hr., and 0.5 mμmol. to 6000 mμmol./hr. To discuss this relationship it is proposed to call the rate of acid formation per unit quantity of initial nicotinic acid, the *apparent catalytic activity* (in mol. titratable acid/mol. nicotinic acid/hr.) of nicotinic acid in catalyzing the formation of acid in the present system. This is a simpler relationship than might have been anticipated in the assay; it is to be expected of a system in which a catalyst (e.g. coenzyme I or II) is present in limiting amounts.

(ii) *Catalytic activity.* Further changes are, however, occurring during the assay. The apparent catalytic activity of nicotinic acid fell with time, from some 12,000 mol./mol./hr. at 18 hr. to 2500 mol./mol./hr. at 72 hr. With the smaller quantities of nicotinic acid the fall in the logarithms of the apparent catalytic activities was linear (figure 8). By extrapolation to the time at which bacterial growth was first considerable ( $t = 16$  hr.) a maximum value for the activity of nicotinic acid as catalyst could be obtained. It is proposed to call this the *catalytic activity* of the acid.

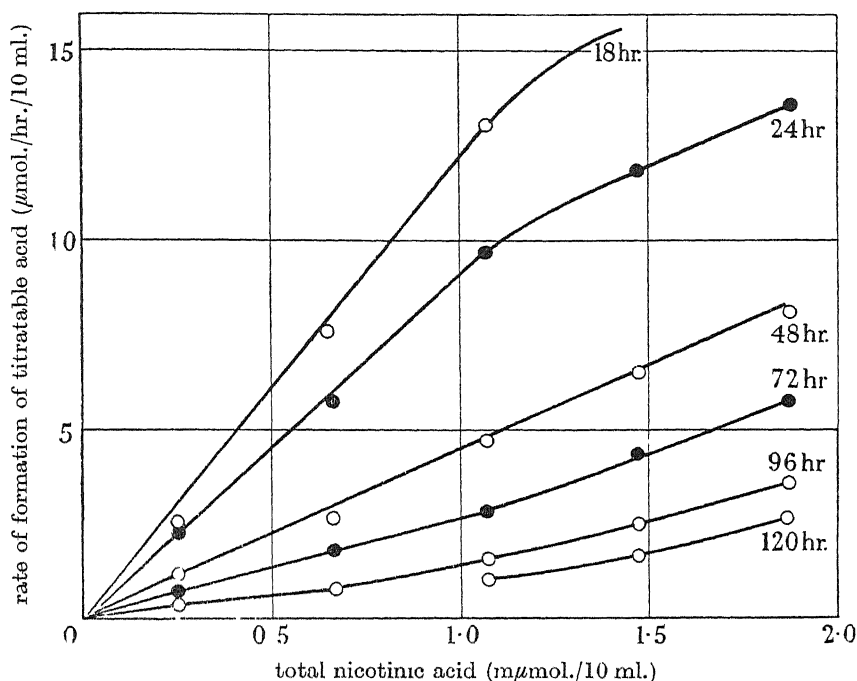


FIGURE 7. Relation between the rate of acid formation at different times (indicated on the figure, in hr.) in the experiment of figure 6, and the nicotinic acid of the culture. The nicotinic acid included that computed from figure 6 to be in the basal medium before addition of nicotinic acid.

## (2) *Changes in nicotinic acid during assay*

### (a) *Loss from cells; the catalytic capacity of nicotinic acid*

As the relationship between added nicotinic acid and rate of acid formation was a simple one in the initial phases of growth, but changed later, it was suspected that the bacterial nicotinic acid itself might be undergoing change with time. To investigate this, a culture of *L. arabinosus* was grown in a batch of the assay medium deficient in nicotinic acid, and samples taken from it at intervals. The cells were separated and their nicotinic acid was determined; the results (table 1) showed loss of two-thirds of the culture's nicotinic acid in 5 days. The course of the loss of nicotinic acid from cells of *L. arabinosus* can be judged from table 1 and figure 4 to be to some extent parallel to the rate of formation of titrated acid, and in fact

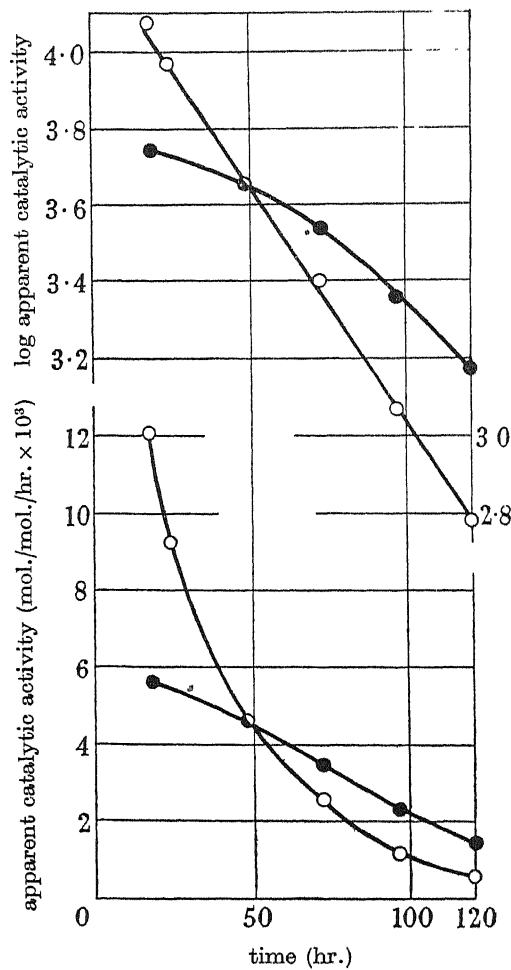


FIGURE 8. Apparent catalytic activities (below) and logarithms of these (above), at different times during the experiments of figure 7. Open circles: 0 to 0.7 mμmol. nicotinic acid/10 ml. Black circles: 1.2 to 1.8 mμmol. nicotinic acid/10 ml.

TABLE 1. LOSS OF NICOTINIC ACID FROM *LACTOBACILLUS ARABINOSUS*

Cells were grown in 100 ml. of assay medium containing 16.3 mμmol. of nicotinic acid added as such, and which gave a blank in assay corresponding to 2.5 mμmol. of the acid/100 ml. At the intervals indicated, 10 ml. portions were taken, the cells separated and assayed for nicotinic acid according to the standard assay method which included a preliminary treatment with acid.

time after inoculation (hr.)	organisms	
	dry wt. (mg./10 ml.)	nicotinic acid (mμmol. in portion from 10 ml. culture)
0	0.1	0.01
24	1.80	1.70
69	2.07	1.06
116	2.14	0.70



a direct proportionality between the two changes was found (figure 9). The ratio mol. titratable acid formed/mol. nicotinic acid lost was relatively stable ( $8.0, 5.2, 6.4 \times 10^5$ ; mean,  $6.1 \times 10^5$ ; see also below), and will be referred to as *the catalytic capacity* exhibited by nicotinic acid.

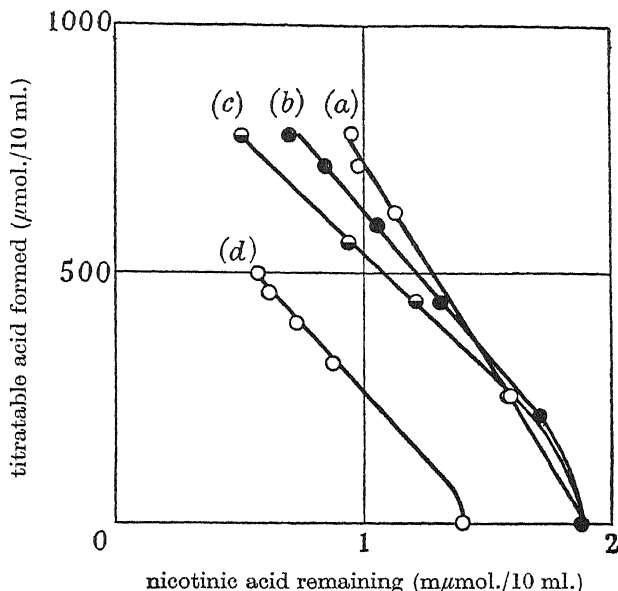


FIGURE 9. Relationship between the quantity of titratable acid formed at different times by cultures of *L. arabinosus* limited in nicotinic acid, and the quantity of nicotinic acid remaining in the cells. Each of the lines (a) to (d) refers to one culture.

### (b) Course of the change

If nicotinic acid remained the limiting factor in acid formation during the period of assay, and continued to have the same catalytic activity, the course of formation of titratable acid would be completely specified and would in general follow a course similar to that of figure 3. This is shown in the following section.

### (3) Quantitative interconnexion between changes during assay

The definition of catalytic capacity  $c$  can be expressed as  $q = q_0 - (Q/c)$ , where  $Q$  = quantity of titratable acid and  $q$  = quantity of nicotinic acid at time  $t$ , and  $q_0$  = nicotinic acid present initially; and that of catalytic activity  $a$  as  $dQ/dt = aq$ . From these can be derived  $\log q = \log q_0 - (a/c) t \log e$ . This indicates, first, that the logarithms of the quantity of nicotinic acid remaining in the assay system, should fall linearly with time. Experimental findings are recorded in figure 10 and are seen to be in approximate agreement with this expectation. The agreement is approximate only, and reasons for this are discussed below. Further, the rate of fall in the logarithms of the quantities of nicotinic acid remaining should be independent of the initial quantity of nicotinic acid. From figure 10 this appears to be substantially the case.

Some of the constants of the last equation may be evaluated from figure 10. The *intercept* at  $t = 16$  hr. (not  $t = 0$ ; see above) should give the logarithm of the initial quantity of nicotinic acid, and this was found to be so. The *slope* gives

$$a/c \log e = 3.3, 3.7 \text{ and } 3.8 \times 10^{-3}, \text{ whence } a/c = 8.3 \times 10^{-3} \text{ hr.}^{-1}.$$

It will be observed that the values for  $a$  and  $c$  derived previously give a ratio of  $1.2 \times 10^4 / 6.1 \times 10^5$ , or  $1.95 \times 10^{-2} \text{ hr.}^{-1}$ . We have confirmed (see below) that the value for  $c$  derived previously holds good during assay; the present ratio therefore suggests a value of  $8.3 \times 10^{-3} \times 6.1 \times 10^5$ , or  $5.1 \times 10^3$ , for  $a$ .

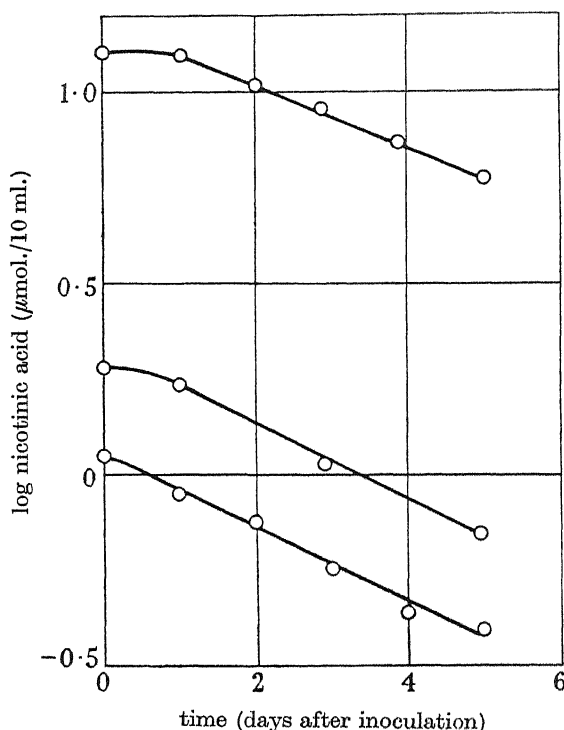


FIGURE 10. Course of the loss of nicotinic acid, shown as logarithms of the quantities remaining in the cells of 10 ml. portions of three cultures, at different times after inoculation.

TABLE 2. ANALYSIS OF FIGURE 11

(A)	(B)	(C)	(D)	(E)	(F)
initial nicotinic acid ( $\mu\mu\text{mol.}$ )	intercept at $t = 16$ hr. ( $= \log Ka$ )	rate of acid formation at 16 hr. (antilog of B; $\mu\text{mol./10 ml.}$ culture/hr.)	catalytic activity of nicotinic acid (C/A; $\text{hr.}^{-1}$ )	slope ( $= a/c \log e$ )	catalytic capacity of nicotinic acid ( $= 0.434 D/E$ ; $\text{mol./mol.}$ )
1.83	1.19	15.5	8,500	$7.6 \times 10^{-3}$	$5.4 \times 10^5$
1.43	1.17	14.8	10,300	$9.0 \times 10^{-3}$	$5.0 \times 10^5$
1.02	1.10	12.6	12,300	$11.4 \times 10^{-3}$	$4.7 \times 10^5$
0.61	0.90	7.9	12,900	$11.4 \times 10^{-3}$	$4.9 \times 10^5$
0.23	0.47	2.9	12,500	$12.1 \times 10^{-3}$	$4.5 \times 10^5$

The first two equations also yield  $\log dQ/dt = \log aq_0 - (a/c)t \log e$ . Figure 11 (from data of figure 7) shows the logarithms of the rate of acid formation to fall linearly with time as is required by this expression; *intercepts* at  $t = 16$  hr. should give  $\log aq_0$  and so afford values for  $a$ . Table 2 gives such values and also values for  $c$  which are obtained from the *slopes* of the lines of figure 11; both are in agreement with those previously obtained independently.

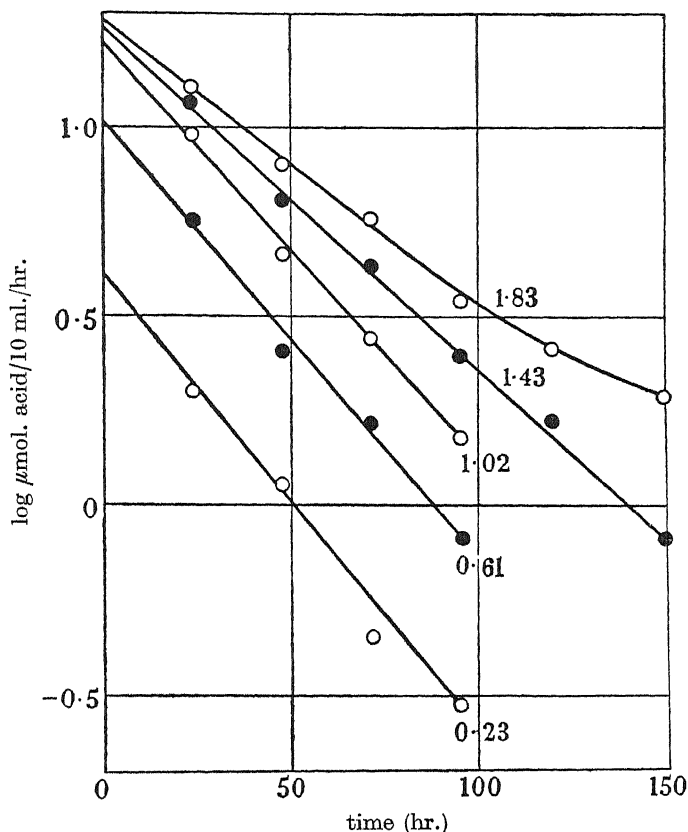


FIGURE 11. Logarithms of the rate of acid formation at different times in the experiment of figure 7. Numbers beside the lines give the quantity of nicotinic acid initially present in 10 ml. of the different cultures (including that computed to be in the basal medium).

#### (4) Variation in catalytic activity

The definition of catalytic activity was based on the rates of acid formation at the beginning of growth; values for  $a$  fell with time and must now be compared with the concomitant fall in nicotinic acid. This has been done in table 3, which shows that acid formation fell at a greater rate than could be accounted for by change in nicotinic acid. At least two factors seemed likely to be involved in this: ageing of the cells, and changing pH following acid production. Variation in catalytic activity with pH was determined as follows. Batches of cells were grown under assay conditions, from limited nicotinic acid, for 2 days. They were then harvested, washed, and used as non-proliferating suspensions. The suspensions were added to

batches of basal medium without added nicotinic acid and buffered at different pH values. The quantities of acid produced under the different conditions were determined in the usual manner but over shorter experimental periods of 2 to 8 hr. The course of acid formation in time was approximately linear, and defined values were obtained for the rates of acid formation. These were about twice as great at pH 6.7 as at pH 5. The quantities of nicotinic acid in the original suspensions were determined by assay of aliquots of the original suspensions of cells, and the rate of acid formation expressed as  $\mu\text{mol. titrated acid}/\mu\text{mol. nicotinic acid}$ , i.e. as catalytic activities. These fell with fall in pH as indicated in figure 12.

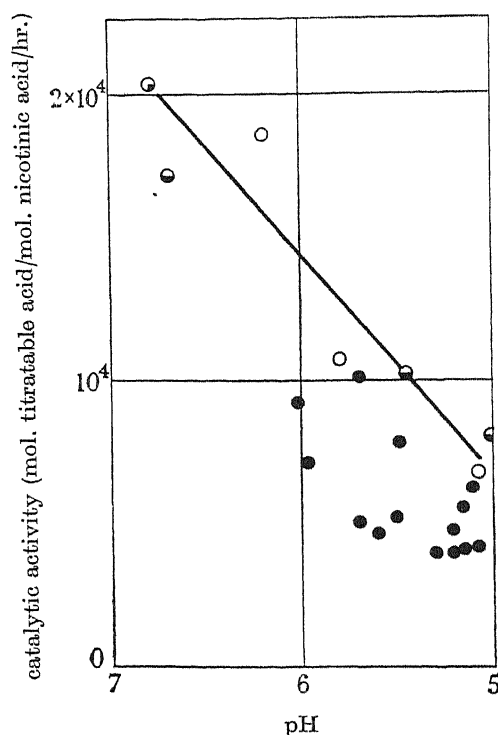


FIGURE 12. Fall in catalytic activity with fall in pH. Upper circles and line: two experiments with cell suspensions prepared after 40 hr. growth. Lower points: derived from several assay cultures in which cells were used between 1 and 6 days after inoculation.

In figure 12 have also been gathered values for catalytic activity of nicotinic acid as determined during assay. These form an irregular group with mean values 0.5 to 0.7 of those given by the resting suspensions at the same pH. Much of the scatter appears due to experimental circumstances which are not strictly controlled during assay. Beyond this scatter it appears that the fall in catalytic activity of nicotinic acid in assay below that of resting suspensions of cells increases with time; this may be attributed to ageing of the cells, which was next investigated.

Rates of acid formation by cells reaped at different times after inoculation were determined manometrically with and without additions of nicotinic acid (table 4). On the first day,  $Q_{\text{CO}_2}$  values (corresponding to  $\mu\text{mol. acid formed/mg. cells/hr.}$ )

of about 15 to 17 were obtained, and these were found to fall with increasing period of growth.

The relation of the cultures to nicotinic acid was also studied by adding nicotinic acid during the manometric experiments with harvested organisms. Acid formation by cultures on their first day was slightly increased by added nicotinic acid; this effect remained slight on the second day also, when excess of nicotinic acid had been added in growing the culture (table 4(b)). In a deficient culture (table 4(a)) stimulation by nicotinic acid increased from day to day; on the second day the increase was to 150 % of the value before addition of nicotinic acid, and on the third day to 280 %. In spite of this, however, the final values for  $Q_{CO_2}$  fell with increasing age of the parent cultures. After 5 days the rate with nicotinic acid was 67 % of its initial value. These observations were made in solutions buffered throughout at pH 7 and therefore represent changes in the acid-forming systems which are independent both of nicotinic acid and of pH.

TABLE 3. DETERMINATION OF CATALYTIC ACTIVITY OF NICOTINIC ACID DURING ASSAY

batch of organisms; initial nicotinic acid (m $\mu$ mol./10 ml.)	time after inoculation (hr.)	rate of formation of titratable acid ( $\mu$ mol./10 ml./hr.)	nicotinic acid of cells (m $\mu$ mol./ 10 ml.)	catalytic activity (mol./mol./hr., $\times 10^3$ )	pH of cultures
(a): 1.45	25	7.0	0.72	9.7	6.02
	48	3.9	0.75	5.2	5.67
	72	3.0	0.55	5.5	5.54
	96	2.2	0.42	5.3	5.43
	115	1.75	0.39	4.5	5.39
(b): 1.88	24	10.8	1.59	6.8	5.92
	44	9.4	1.20	7.8	5.50
	72	5.2	1.13	4.6	5.22
	96	3.9	0.98	4.0	5.12
	113	3.9	0.98	4.0	5.07
(c): 1.88	24	20	1.74	11.5	5.71
	43	5.5	1.05	4.9	5.36
	72	3.15	0.81	3.9	5.25
	96	2.51	0.60	4.2	5.18
	120	2.10	0.36	5.8	5.14
	168	1.71	0.27	6.3	5.07

A further point remained to be decided before the preceding interpretation of table 4 was fully satisfactory. Nicotinic acid presumably exerted its catalytic effect in acid formation as a derivative such as cozymase. Growing cells could then synthesize cozymase very effectively from nicotinic acid, but it remained possible that older cells could not do so. Table 5, however, shows that cozymase synthesis was adequate at even 7 days. (It is to be emphasized that this observation refers to experiments at pH 7, and not at the pH of about 5 which would be attained in assay after 7 days. Synthetic ability at pH 7 is relevant to the interpretation of table 5, and gives the data necessary for concluding that acid-forming

systems deteriorate during assay. Synthetic ability at various pH values is relevant to the assay as a whole, and has been investigated; it will be reported elsewhere.)

TABLE 4. EFFECT OF NICOTINIC ACID ON RATES OF ACID FORMATION  
BY CULTURES OF *LACTOBACILLUS ARABINOSUS*

Cells were grown in batches of assay media initially containing the quantities of nicotinic acid specified below. At the times indicated the cells were harvested, washed with saline and suspensions equivalent to 2 to 3 mg. dry wt. added to Warburg vessels containing glucose (150  $\mu$ mol.), NaHCO<sub>3</sub> (80  $\mu$ mol.), and the inorganic (but not ammonium) salts of the growth medium, in a final volume of 3 ml. Nicotinic acid (4  $\mu$ g.) was contained in a side arm and the whole equilibrated with 5% CO<sub>2</sub>-N<sub>2</sub>. In several cases lactic acid was determined chemically and found to account for 92 to 95% of the CO<sub>2</sub> evolved. Nicotinic acid, nicotinamide and cozymase were found to have similar effects in the instances examined (which did not however include limiting quantities of the substances).

nicotinic acid of culture (M $\times 10^7$ )	age of culture (hr. after inoculation)	Q <sub>CO<sub>2</sub></sub> between 10 and 60 min. after mixing ( $\mu$ mol./mg./hr.)	
		without addition	with nicotinic acid or derivative
(a): 0.92	19	15.2	17.0
0.92	46	9.2	12.9
0.92	115	4.1	11.4
(b): 4.9	19	16.8	17.4
4.9	46	13.9	14.8

TABLE 5. CHANGE IN RATE OF ACID PRODUCTION FROM GLUCOSE  
BY ORGANISMS HARVESTED AT DIFFERENT TIMES DURING ASSAY

A 300 ml. culture of *L. arabinosus*,  $1.63 \times 10^{-7}$  M with respect to nicotinic acid, was grown for 40 hr. in the assay medium and a 100 ml. portion separated to give the 2-day-old cells. To half of this while still sterile, nicotinic acid was added to make the solution  $1.3 \times 10^{-6}$  M and the suspension left for 3 hr. at 37° C. The cells from this and from the untreated half were reaped, washed once with 0.9% NaCl and added to bicarbonate saline with 0.07 M glucose, in Warburg vessels. These contained either cozymase or nicotinic acid, to make final concentrations of  $4 \times 10^{-6}$  M, in side arms. After determining the initial rate of CO<sub>2</sub> evolution, without addition, the nicotinic acid and cozymase were added. Response to the substances added was completed within 10 min. and the new rates were determined during the next 20 min.

After 7 days' growth, a similar experiment was performed with the remainder of the culture.

substance added to organisms ( $\mu$ mol.)	time of exposure to substance (min.)	Q <sub>CO<sub>2</sub></sub> at		
		18 hr.	2 days	7 days
none	—	17.1	8.3	1.5
nicotinic acid	10	—	10.2	2.1
nicotinic acid	180	—	10.9	4.0
cozymase	10	—	13.1	2.2
nicotinic acid	180	18.0	15.0	3.9
followed by cozymase	10			

The restoration of acid formation was brought about by concentrations of nicotinic acid which were higher than those available during assay, and even after the restoration (table 4) nicotinic acid did not exert its full catalytic activity. The extent to which the catalytic activity had fallen was assessed as follows. After

7 days, 31% of the original nicotinic acid remained in the cells of figure 10. Glycolysis by organisms on the 7th day after inoculation and following a short incubation with nicotinic acid was found to be 12.2% of the value at 24 hr. The cells as they existed on their 7th day were therefore such that the remaining nicotinic acid could exert only 40% of its full catalytic activity. It actually exerted less than this value, and only 27% of that which would initially have been exerted by the quantity of acid remaining. This is to be compared with an observed fall in catalytic activity of from 12.5 to  $3.5 \times 10^4$ , or to 28% of the initial value. The manometric and growth experiments therefore give consistent pictures of the falling catalytic activity of nicotinic acid.

Beyond the fall in acid formation due to change in pH and to loss of nicotinic acid, changes due to decay in other systems concerned with glycolysis can therefore be discerned. This is understandable, as the type of relationship at present observed between glycolysis and loss of nicotinic acid exists also between glycolysis and other vitamin-like substances (McIlwain 1947).

## DISCUSSION

### (1) *Nicotinic acid derivatives as catalysts*

Two new terms—catalytic activity and catalytic capacity—have been employed in the present study, and their use requires assessing.

(a) *Catalytic activity.* The catalytic activity, or mol. titratable acid formed/mol. nicotinic acid/hr., has the form of an enzyme turn-over number. The acid titrated is largely lactic acid, and the nicotinic acid of *L. arabinosus* exists largely as cozymase (McIlwain, Stanley & Hughes 1948). Although a turn-over number would be applicable in describing, for example, the activity of triosephosphate dehydrogenase, isolated from cells, towards 3-phosphoglyceraldehyde and cozymase (that from yeast catalyses the reaction at the rate of some  $10^6$  mol./mol./hr.: Warburg & Christian 1940), this turn-over number would not immediately be applicable to the dehydrogenase as it exists in the cell. There the pH and substrate concentrations are defined physiologically; for a turn-over number they are such as to permit maximal enzyme activity. Also, the enzyme turn-over number would not be the same as the number of molecules of 3-phosphoglyceraldehyde caused to react per unit time per molecule of cozymase available. For although the cozymase can be regenerated and so is in effect a catalyst as well as a substrate, it may be present in molar concentrations which differ considerably from those of the dehydrogenase protein. It is to describe the activity of such a system in terms of cozymase that the term catalytic activity has been applied.

The values found for the catalytic activities of nicotinic acid in normal (i.e. not aged) cells of *L. arabinosus* during assay and in related conditions were between 0.4 and  $1.4 \times 10^4$  mol./mol./sec. They are therefore numerically much smaller than the turn-over number of the somewhat comparable enzyme which has been quoted above. The turn-over number and catalytic activities in the two cases refer to different organisms, but in general terms a catalytic activity determined under the conditions of assay can be expected to be less than the potential turn-over

number of a corresponding enzyme. In assay, the system is necessarily sub-optimal in coenzyme concentration, and cozymase exists partly as its dihydro-compound.

(b) *Catalytic capacity*. A given quantity of nicotinic acid yielded only a certain quantity of titratable acid before being lost to the system; the molecular ratio between these two quantities was called the *catalytic capacity* of nicotinic acid in the assay system. Its value ( $c. 5 \times 10^5$ ) was found to be independent of small changes in pH or of relatively large changes in the rate of acid formation. It can be regarded also as representing a ratio between the rates of two processes: those of acid formation and those whose net result is loss of nicotinic acid from the bacterial cells. Its relative constancy suggests that formation and loss of nicotinic acid are connected in some way beyond that ordinarily implied by the fact that nicotinic acid forms part of the acid-producing systems of the cells.

Similar phenomena have been reported in certain other systems, by Lwoff & Lwoff (1937), Lennerstrand (1941), McIlwain & Hughes (1944) and by McIlwain (1946). Lwoff & Lwoff (1937) suggested in explanation of such behaviour that the coenzymes, not being perfect catalysts, suffered a 'wrong' type of reduction and reoxidation in hydrogen transport. The following is a more general way of regarding such phenomena. Cozymase is not entirely stable, though its spontaneous decomposition at pH values between 5 and 7 and at 37° C is much slower than the observed loss in nicotinic acid during acid formation by *L. arabinosus*. But during acid formation it exists partly as a complex with triosephosphate dehydrogenase, and partly as dihydrocozymase and as its complex with lactic dehydrogenase. The proportions of cellular cozymase which exist in these and other forms will vary with the metabolic activities of the cell. The stabilities of cozymase in the different forms are unlikely to be the same. If glycolysis causes a greater proportion of cozymase to be in a less stable form, then glycolysis will be expected to be accompanied by a loss of cozymase which is to some extent proportional to the progress of glycolysis itself. Experiments with dihydrocozymase in buffer solutions over the pH range of the assay have suggested it to be less stable than cozymase but more stable than required to account for the loss in assay.

## (2) *The assay*

(a) *Partial catalytic capacity*. Under the conditions chosen for assay, quantities of titratable acid are produced which are about proportional to the quantities of nicotinic acid added. This regularity expresses the same type of relationship as is expressed by the catalytic capacity of nicotinic acid; but whereas the catalytic capacity of nicotinic acid is the total quantity of titratable acid which can be produced during the consumption of a given quantity of nicotinic acid, the assay curve refers to a lesser quantity of titratable acid and represents only a partial catalytic capacity of the nicotinic acid added. For the standard assay curve to remain linear, a constant fraction of the catalytic capacity of each of the varying quantities of added nicotinic acid, must be used. It is easy to obtain conditions in which this is not the case. If the standard tubes of an assay are titrated on their first day of growth, the quantity of titrated acid does not increase as rapidly as does



the added nicotinic acid (figure 1). A similar response is obtained when quantities of nicotinic acid are employed which are larger than those of figure 1 and when media or other conditions of growth are less satisfactory.

(b) *Self-compensation in assay.* Figure 1 illustrates how during an ordinary assay the partial catalytic capacity of the nicotinic acid which is used in acid formation is at first variable, but after 3 days it becomes a constant fraction of the whole. This change is an essential part of a satisfactory assay. It is not to any major extent due to further growth of the bacteria (figure 3), but can be understood in terms of the defined catalytic capacity of nicotinic acid. For a lesser initial formation of titratable acid leaves in the cells a greater quantity of nicotinic acid, and so allows them a greater rate of formation of titratable acid during the later period. The assay system is thus self-compensating in a manner which would not obtain if nicotinic acid were not being lost from the cells during its progress. It is presumably as a result of such compensation, that variation may occur in the quantity of bacterial growth without detriment to the assay, and also that the assay does not require very accurate control of temperature.

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# Contractile tissues in the mammary gland, with special reference to myoepithelium in the goat

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[Plates 1 to 4]

The distribution of myoepithelium and smooth muscle in the lactating mammary gland of the goat has been examined by a new technique of silver impregnation which is applicable to sections up to  $100\mu$  in thickness. Myoepithelium covers the stromal surface of the epithelium of the alveoli, ducts and cisterns of the entire gland, and is thus much more abundant than is generally realized. Smooth muscle forms scattered inter-lobular bundles closely associated with the blood vessels. The theory that myoepithelial contraction is the principal factor concerned with 'let-down' and the ejection of milk is examined; other factors such as inter-lobular smooth muscle contraction, vascular changes, and elastic recoil of the stroma appear to play minor roles, if any, in this phenomenon. Hitherto, it has been assumed that myoepithelial cells are contractile because they bear structural resemblances to smooth muscle fibres. With the new technique structural changes have been found in the myoepithelium of contracted as compared with distended alveoli and ducts. These changes, together with the general orientation of myoepithelial cells, and the precise relationship between these cells and the folds in the secretory epithelium from contracted glands, are consistent with the assumption that myoepithelium is the contractile tissue in the mamma which responds to a neurohormonal mechanism involving oxytocin.

## INTRODUCTION

In recent years there has been renewed attention to the mechanism by which milk is removed from the mamma in response to suckling, or artificial hand- or machine-milking. The secretion is held by capillary attraction in the alveoli and fine branches of the duct system and some contractile force appears to be indispensable for efficient emptying of the glandular tissue. Suction or manual compression will remove milk stored in the larger ducts and cisterns, but cannot withdraw the large proportion of the total milk-yield confined within the glandular lobules.

Evidence of the presence of actively contractile tissue comes from two main sources. There is firstly the sudden rise in milk-pressure which occurs within an udder at the time of milking (Tgetgel 1926), a pressure in the cow of about 25 mm. Hg; this is evoked by sensory stimulation of the teat, or even through auditory or visual pathways. As yet, no generally agreed scientific term has been adopted for describing this phenomenon: the animal is said to 'let-down' its milk. Secondly there is the well known response of the mammary gland to posterior-pituitary extracts which, on injection into the intact animal, or perfused through the isolated organ, cause a strong contraction sufficient to eject the milk from the teat. A critical survey of the experimental evidence and its interpretation appears in the recent review by Folley (1947).

It is sufficient to mention here that 'let-down' has been regarded in the past either as a purely nervous reflex acting upon smooth muscle in the mammary stroma (Gaines 1915), or a vascular reflex leading to 'erection' of the udder tissues

(Hammond 1936). More recently Ely & Petersen (1939, 1941) have suggested that a neuro-hormonal mechanism involving oxytocin more closely fits the experimental evidence they obtained in the intact animal; evidence which was later to be confirmed by Petersen & Ludwick (1942) in experiments on the isolated organ. With all these theories there is confusion as to the identity and location of the possible varieties of contractile tissue in the mamma. The problem is complicated by the presence of true smooth muscle in the stroma and of myoepithelium in the walls of the alveoli and ducts. In some accounts there has been a tendency to ignore the histological distinction between these two components, as though the term smooth muscle will suffice to describe contractile tissue wherever it may be located. Unfortunately the term myoepithelium means little more than a collection of cells of epithelial origin which have some of the structural appearances of smooth muscle. Their contractile function has been assumed, not proved, probably because they happen to be so much less accessible to experimentation than smooth muscle; much less so than the various types of cells found on the walls of capillaries.

The present paper describes the myoepithelium and smooth muscle in the goat mammary gland. These cells have been outlined by a new technique, which shows more adequately than previous methods the great quantity of myoepithelium present in the lactating gland, and its orientation with respect to the different parts of the mammary lobule. On the assumption that myoepithelium is a contractile tissue, comparisons have been made between the structural appearances of these cells in material containing alveoli and ducts fixed in a state of distension, and while collapsed following withdrawal of the secretion.

#### MATERIAL AND METHODS

The material available for this investigation was from four British Saanen goats in full lactation following a normal pregnancy. In fixing the mamma for histological examination, it was essential as far as possible to preserve the glandular alveoli and ducts in a state of distension or collapse produced respectively by allowing the gland to remain un milked for the previous 24 hours, or by milking it out as completely as possible just before fixation. If a distended gland is cut into small blocks before fixation it collapses, and the alveoli become distorted and irregular. Fortunately each half of the goat's udder is supplied by a separate single artery (external pudic) and these arteries offer the most favourable conditions for a complete perfusion with fixative of the two glands of the udder. After a short washing out with normal saline containing a trace of sodium nitrite, the fixative was perfused until it flowed from the two main veins draining the gland. Then the whole gland, with its teat and cisterns intact, was immersed in fixative, taking care to seal up any small accidental perforations leaking milk from the cisterns. After several days' immersion in fixative, the gland was sliced horizontally into slabs 2 cm. thick. The uncoagulated milk was washed off with tap water, and the tissue exposed to the fixative for a further period, or stored in 10 % formol-saline. Fixation with Zenker-formol was chosen for routine examination because it carried the advantage that small blocks could be isolated for subsequent osmic impregnation of fats by the method of Hoerr (1936).

A special technique was devised for silver impregnation of myoepithelium and smooth muscle in thick sections. This was discovered accidentally while experimenting with suitable methods of nerve impregnation in the lactating gland. The technique, as used, is probably unnecessarily elaborate, and should bear modification and improvement, particularly with respect to simplification of the initial fixing solution. The method is best described in the following stages:

1. Fixation by perfusion with a fixative devised by Weber (1944).

dioxane	450 ml.
isopropyl alcohol	450 ml.
commercial formalin	200 ml.
formic acid	20 ml.
3 % aq. cobalt nitrate	100 ml.
chloral hydrate	50 g.
glacial acetic acid	10 ml.

The whole gland is immersed in the fixative for 2 to 3 days, then cut into slabs and stored in the fixative for a further period of 1 to 4 weeks.

2. Small blocks cut from the surface of a slab are washed in running water overnight.

3. Frozen sections are cut at 50 to 100 $\mu$  without preliminary soaking of the tissue in gum or gelatine. A thickness of 75 $\mu$  was suitable for showing large clusters of alveoli mainly in surface view and in continuity with intra-lobular ducts.

4. The sections are collected direct from the knife into an acetate buffer solution made from N/10 NaOH and N/10 acetic acid at pH 5.2 to 5.4.

After transferring the sections through several dishes containing fresh buffer, in which it was found that they could remain as long as overnight if required, they are carried through a variant of the Cajal method for frozen sections (see McCullung, 1937).

5. Transfer sections from buffer solution into:

10 % aq. silver nitrate	3 ml.
dist. water	5 ml.
abs. ethyl alcohol	0.5 ml.
pyridine (pure)	4 drops
(50 drops = 1 ml.)	

The above components of the silver bath are set out in this way to facilitate variation of their concentrations for individual batches of sections, if required.

The sections are placed immediately in an incubator at 55° C for 10 min.

6. Rinse individual sections for 5 sec. in abs. ethyl alcohol. The same bath can be used for a dozen or more sections.

7. Reduce in:	hydroquinone	0.3 g.
	neutral commercial formalin	30 ml.
	dist. water	70 ml.

8. Wash in dist. water, and fix in 5 % aq. hypo.

9. Attach the sections to gelatinized slides, using formalin vapour, dehydrate, and mount in Canada balsam.

As with all methods of silver impregnation, it is useful to record some of the vagaries of the technique encountered in its use. The method is not unduly capricious because it has been repeated on four glands, each being fixed and carried through on different occasions. It would require careful investigation, however, with numerous variations of the method to establish precisely how the various stages in the method could be simplified, or more accurately controlled. The following points seem worth recording. Fixation for periods over one week intensifies the silver staining of the entire section but does not abolish the relative specificity for myoepithelium. Prompt treatment with hypo is necessary to stop the reduction of silver in over-fixed material; this is better than toning the section with gold chloride. Under-fixed material does not give the reaction, however one may attempt to modify the silver impregnation. The concentrations of silver and alcohol in the impregnating bath are not critical. If the pyridine is impure its concentration may have to be increased from 4 drops to as much as 1 ml. Whatever modification of the impregnating solution and reducer was tested it was clear that the specificity of the reaction for myoepithelium is conditioned above all by the transition of the section from an acid medium into the alkaline silver bath. No experiments were carried out on material with different initial fixation except for one specimen fixed in simple formalin in which the reaction failed entirely. Post-mortem human lactating breast, fixed by immersion in Weber's fluid, gave some indication that the reaction would be successful but insufficient material has so far been available to achieve results comparable with those from the goat glands.

#### OBSERVATIONS

##### *The identification of myoepithelium in thin sections by routine staining.*

It is proposed to confine the term myoepithelium to elongated cells lying in close contact with the epithelium of the ducts and alveoli of the lactating gland. The immature stages of these cells, which in the opinion of Benda (1894), Retterer & Lelièvre (1911) and most other histologists can be traced back through the growth phases during pregnancy to the outer layer of cuboidal epithelium on the ducts of the resting gland, are potential myoepithelium, but such cells are unlikely to be contractile whatever other functions they may possess. Typical smooth muscle exists in bundles surrounded by connective tissue in the stroma, or in its usual location in the walls of blood vessels. Whether such an arbitrary separation is justified, on grounds other than derivation from ectoderm or mesoderm, will depend ultimately on whether separate activities in response to hormonal or nervous stimulation can be ascribed to the two varieties of cells.

Thin sections of lactating gland, fixed in Zenker-formol while distended with milk, and stained with Heidenhain's iron haematoxylin and orange-G-erythrosin, show elongated cells arranged at intervals over the stromal surface of the alveoli and ducts. Some are spindle-shaped, others show branching processes extending

from a central cell-body containing the nucleus, but only a hazy idea of the shape of these cells can be obtained from thin sections. Thus in longitudinal section (figure 1, plate 1) the nucleus is easily distinguished as being paler and larger than the nuclei of the secretory epithelial cells. Processes of the cell can be traced for some distance in contact with the epithelium, because their cytoplasm is acidophil in contrast to the basophil cytoplasm of the secretory cells. A faint longitudinal striation has been observed. In tangential section the central areas of the cells stand out in contrast to the mosaic of underlying secretory cells (figure 3, plate 1). When cut transversely the processes of the myoepithelial cells can be identified as small oval or triangular areas (figure 2, plate 1), not only in contact with the secretory epithelium but actually indenting individual cells so that in places the myoepithelial process appears to be partly surrounded by prolongations of the basophil cytoplasm of the secretory cells. The endothelium of blood capillaries, dilated by perfusion with the fixative, is separated from the alveolar wall by sparse areas of connective tissue, and there can be no question of confusing endothelium, fibroblasts or reticular fibres with the myoepithelial cells.

It will be realized that there is considerable difficulty in outlining these cells with greater precision in the lactating gland in a collapsible tissue full of secretion, or of studying them in the living state, or testing them for birefringence, localization of enzymes, and other cytological characters. Their cross-sectional area is minute. A wax model was constructed from serial  $5\mu$  collodion-paraffin sections of a strip of alveolar epithelium with transversely sectioned myoepithelial cells to check up some finer details. This showed that the ends of the main processes of the cells branched into minute terminal endings approaching  $1\mu$  in thickness. Contact between the processes of separate cells occurred within the thickness of a single section, but it was impossible to establish whether the cells formed a true syncytial network.

*Orientation and distribution of myoepithelium as seen in  
thick, silver-impregnated sections.*

The method of silver impregnation outlines the myoepithelium in sections up to  $100\mu$  in thickness (figure 4, plate 2), whereas aniline dyes fail to differentiate these cells in similar sections owing to the great staining intensity of the secretory epithelium and stroma. In using silver there was always the danger of confusion with connective tissue fibres (figure 14, plate 3), but it was found that the technique could be adjusted to give fairly sharply outlined myoepithelial cells showing pale nuclei, exactly comparable in size and position with those already observed in the routine preparations. Moreover, smooth muscle fibres in blood vessels were stained to about the same intensity (figure 7, plate 2), leaving the connective tissue stroma clear, at least in the areas immediately adjacent to the alveolar walls. In some variants of the technique only the blood capillaries were stained in addition to the mammary epithelium. These preparations provided a check on the vascular networks, which are quite unlike the myoepithelium in the pattern they form on the alveolar walls (figure 13, plate 3). While the identification of the silver-stained cells as myoepithelium is beyond doubt, it must be admitted that the impregnation is

granular, and there is some doubt whether the finer processes of the cells are reliably shown (figure 12, plate 3). The silver technique gives an impression of the distribution and orientation of these cells rather than providing details of their minute structure.

The myoepithelium is invariably longitudinal in arrangement on the walls of the intra-lobular ducts (figure 6, plate 2). At the point of junction with alveoli, which in the mammary gland is indistinct as far as the lining epithelium is concerned, the myoepithelial cells sweep across onto the alveolar wall without discontinuity. The orientation of the alveolar myoepithelium is distinctly longitudinal in the larger alveoli, which are usually pear-shaped. A few irregularly disposed cells are located at the blind ends of these alveoli. The smaller, more spherical alveoli are surrounded by whorls of myoepithelium of somewhat confused pattern.

There is a lining epithelium usually two cells in thickness forming the walls of the inter-lobular ducts and cisterns. In these regions it was more difficult to obtain clear-cut impregnation of myoepithelium, possibly because the underlying connective tissue was denser. The best preparations were from a distended gland. These showed that the duct system and cisterns have a myoepithelial layer, if anything slightly denser than that on the alveoli (figures 9 and 10, plate 3), and sufficient sections were available to conclude that the entire duct and cistern epithelium is probably covered with these cells. In the contracted ducts the epithelium is thick and folded making the identification of myoepithelium less precise owing to possible confusion with reticular fibres (figure 11, plate 3).

#### *Smooth muscle*

Bundles of smooth muscle were scattered irregularly in the connective tissue septa between the lobules (figure 8, plate 2). They were usually surrounded with areas of collagen, and rarely came into contact with the marginal alveoli of a lobule. The outer capsule of the gland, in continuity with the overlying skin, lacked smooth muscle almost entirely. Within the lobules there was no smooth muscle interspersed between the alveoli or following the course of the intra-lobular ducts; nor had the larger ducts any muscular coat or sphincters. It was somewhat surprising to find how sparse the smooth muscle was in association with the walls of the cisterns. Figure 9, for instance, shows no muscle bundles at the entrance of a duct into a cistern, nor in the wall of the cistern itself. The greatest concentration of smooth muscle was often found in close contact with the major blood vessels. The veins in particular were surrounded by a palisade of muscle bundles coursing longitudinally with each vessel; in places the smooth muscle fibres in the wall of a vein splayed out to mingle with the inter-lobular bundles. It was clear from examination of sections at numerous levels in the glands that the total concentration of smooth muscle was altogether too slight, and its distribution too irregular, for it to play a major role in squeezing the milk from the alveoli into the duct system.

#### *The appearance of myoepithelium in distended and in contracted alveoli*

If it is assumed that myoepithelial cells are the important contractile elements of the mammary lobules it is reasonable to suppose that they will become passively

stretched out on the alveolar walls during the phase of distension, when milk is being secreted into the alveoli. Figure 5, plate 2, of a large alveolus shows how elongated these cells can actually become. Then, at the moment of 'let down' the cells should develop a state of tension so that, when sufficient milk is removed from the cisterns, the secretion in the alveoli and ducts is pressed along to take its place. It seems essential, if there is to be maximum evacuation of the stored milk from the lobular tissue, that the contraction phase of the myoepithelium should persist until the alveoli are completely collapsed. Any structural change in the myoepithelium occurring as the result of contraction would hardly be detectable in the initial phase of tension at the onset of 'let-down'. All that could reasonably be expected would be to see a shortening and thickening of the cells at the end-point of contraction, when the alveoli were empty, and to find that the contractile cells remained taut, and bore some intelligible relationship to the folds produced in the alveolar epithelium.

To test these assumptions, the following experiments were undertaken:

(a) A gland was removed from a goat while distended full of milk, the teat cannulated, and a water manometer connected to measure the milk-pressure within the cisterns. Perfusion through the artery with saline, and then with fixative, caused no rise in milk-pressure if the perfusion was carried out at low pressures. Higher pressures, equivalent to about four times the normal blood pressure, gave a rise of about 1 cm. of milk-pressure. It was concluded that fixation by perfusion did not cause any appreciable contraction in the mammary tissue comparable with that which occurs during 'let-down'.

(b) A comparison was now made between the histological appearance of one half of an udder fixed in a state of maximum distension, and the remaining half from the same goat, milked out as completely as possible before fixation. The practice of previous workers has been to isolate small blocks of lactating mammary gland for immersion in fixative, which inevitably leads to collapse and distortion. No attempt appears to have been made to carry out the above simple experiment, and so to examine the structural changes occurring in alveoli under reasonably controlled conditions in the same animal. However, the experiments of Schäfer (1915) on the cat amount to much the same thing. He showed that pituitrin produced a collapsed state of the alveoli of an individual gland with an excised nipple, whereas the adjacent glands with intact nipples remained distended.

The main conclusions from the goat material (figures 15 and 17, plate 4) were that the lobules shrink as a whole when the gland is emptied by milking, and the inter-lobular septa are widened to the extent of being easily visible to the naked eye in a large horizontal section through the complete gland. The alveolar epithelium thickens about three-fold, its cells becoming columnar in shape and projecting irregularly into the lumen. The alveolar walls often become wrinkled, particularly in the larger alveoli, which become very elongated with a cleft-like lumen. Smaller alveoli remain approximately spherical. The ducts remain open but they are much narrower than in the distended gland with at all times a complete continuity of lumen between the ducts and the cisterns. The contracted ducts have thicker and more folded walls.



*The myoepithelium as seen in thick, silver-impregnated sections  
from a distended and a collapsed gland*

There is considerable variation in the shapes and sizes of myoepithelial cells covering the alveoli in any particular lobule, whether distended or contracted. If all the cells were uniformly spindle-shaped it would not be difficult to measure them, and so to detect a shortening and thickening in the contracted state. Examination of a large number of sections from distended and contracted material showed that the cells on the whole were more elongated on distended alveoli, and more branched and star-shaped on contracted ones (figures 16 and 18, plate 4). The number of branching processes appeared considerably greater in the contracted gland. However, for reasons which will be discussed later, it was not considered practicable to undertake detailed measurement of the cells in the two glands.

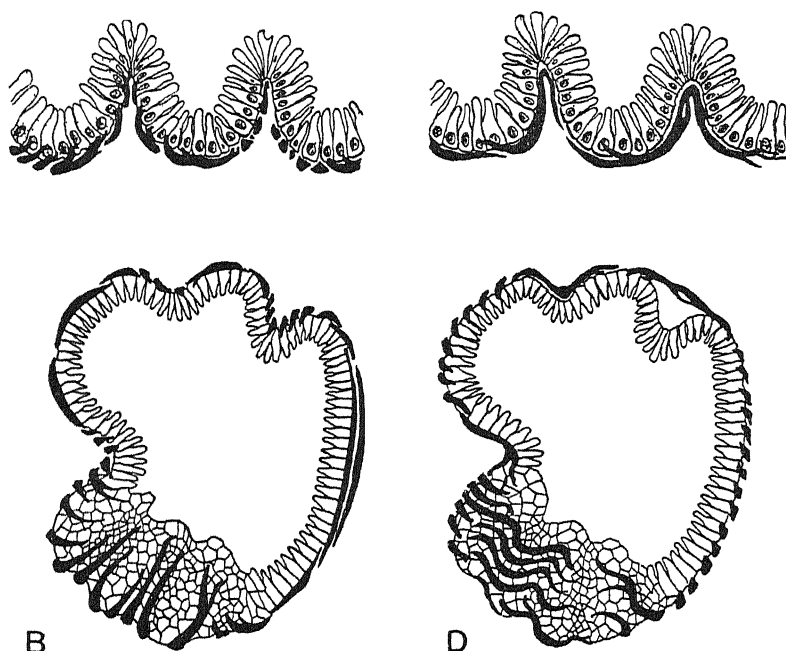


FIGURE 19. Drawings to illustrate the relations of myoepithelial cells to folds in alveolar epithelium of a contracted gland. A and B, the relationship found in the actual material; C and D, a hypothetical relationship based upon the assumption that the myoepithelial cell is non-contractile, and is passively folded with the overlying secretory epithelium when the alveolus collapses.

The precise relationships between myoepithelial cells and folds in the contracted alveolar epithelium offer perhaps the clearest indication that myoepithelial contraction does take place. It is difficult to visualize in three dimensions how the contractions of several elongated cells might combine to produce a fold of irregular shape in a particular part of an alveolar wall. Suppose, however, a strip of epithelium (figure 19A) is thrown into two fairly sharp, parallel folds. It was found quite consistently in the sections from the contracted gland, that the myoepithelium was cut transversely or obliquely in such areas, with the pointed ends of individual

cells reaching up into the crests of the folds. This indicated that the deep folds were associated with myoepithelial cells orientated parallel to the fold, or converging obliquely towards its margins. Figure 19B shows an entire cross-section of an alveolus with the lower part of the wall cut obliquely in surface view. There are three folds showing this longitudinal orientation of the myoepithelium. Note on the right a long strip of unfolded epithelium accompanied by longitudinally cut, tautly stretched myoepithelium. At the top the cells are again cut transversely and obliquely in association with two more folds. Such relationships were frequently

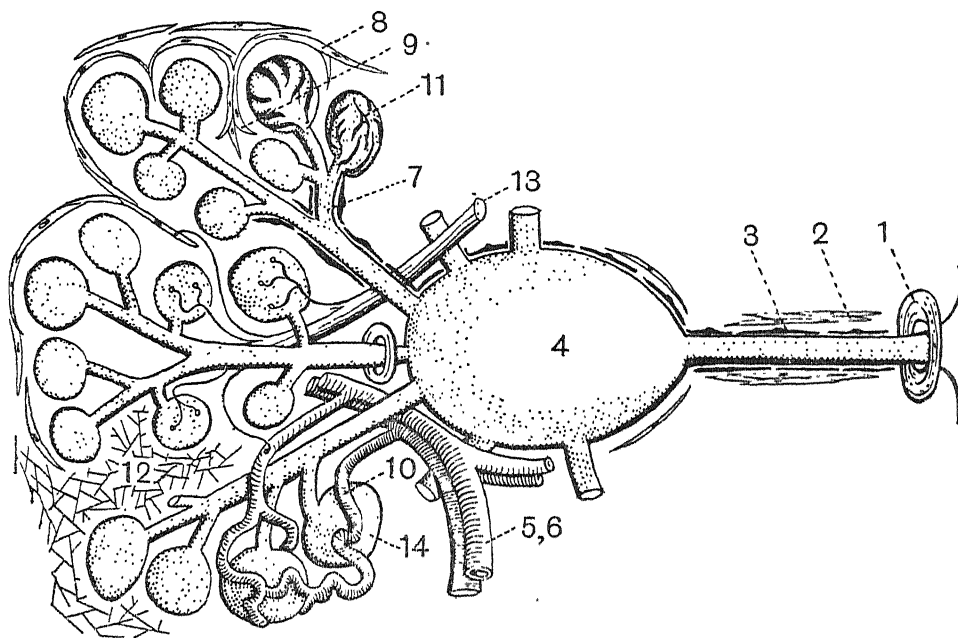


FIGURE 20. Diagram of a generalized exocrine gland showing the location of possible factors influencing flow of secretion.

*Duct activity influenced by*—1, smooth muscle sphincters; 2, longitudinal smooth muscle shortening ducts or producing peristalsis; 3, myoepithelium; 4, reservoirs in large ducts, or cisterns; 5 vasodilatation pressing on ducts or reservoirs; 6, vasoconstriction shortening inter-lobular vessels and squeezing adjacent ducts; 7, secretion from duct epithelium.

*Lobule activity influenced by*—8, smooth muscle bundles in inter-lobular septa squeezing lobules as a whole; 9, smooth muscle interspersed between alveoli; 10, vasodilatation or vasoconstriction affecting alveoli mechanically; 11, myoepithelium; 12, elastic fibre recoil in stroma when pressure in distended alveoli is released; 13, nervous stimuli to secretory epithelium and smooth muscle; 14, hormonal stimuli to epithelium.

observed in the larger alveoli and ducts of the contracted material. On the other hand if myoepithelial cells are non-contractile, like fibroblasts, in some of the folds at least one would expect to find a relationship similar to figure 19C, where the cell is cut longitudinally, with its processes bent in a passive manner to follow the contours of the fold. Seen in surface view in a section through a complete alveolus (figure 19D) such cells would be wavy in outline as they lay across the folds. They were never like this. In fact all the myoepithelial cells shown in figures C and D are arranged in a direction contrary to that found in the actual material. The cells do

not, for instance, lose contact with the alveolar epithelium to bridge across the margins of a fold (figure 19D), which is what one would expect if contraction of a cell orientated at right angles to the axis of a fold actually occurred. The myoepithelium is tautly contracted on the collapsed alveoli, or stretched out into very elongated cells on distended alveoli. It never loses contact at any point with the surface of the alveolar epithelium when contraction occurs. Folds in this epithelium follow the direction of the main processes of the myoepithelial cells.

### DISCUSSION

The factors which are known, or supposed on experimental grounds, to influence the flow of secretion from exocrine glands in general may be summarized in a diagram (figure 20). It will be convenient to examine these factors not only in regard to the mamma but also in the other glands in which myoepithelium has been identified. There is a broad distinction between activity associated with the main ducts, and activity occurring at the site of the secretory units.

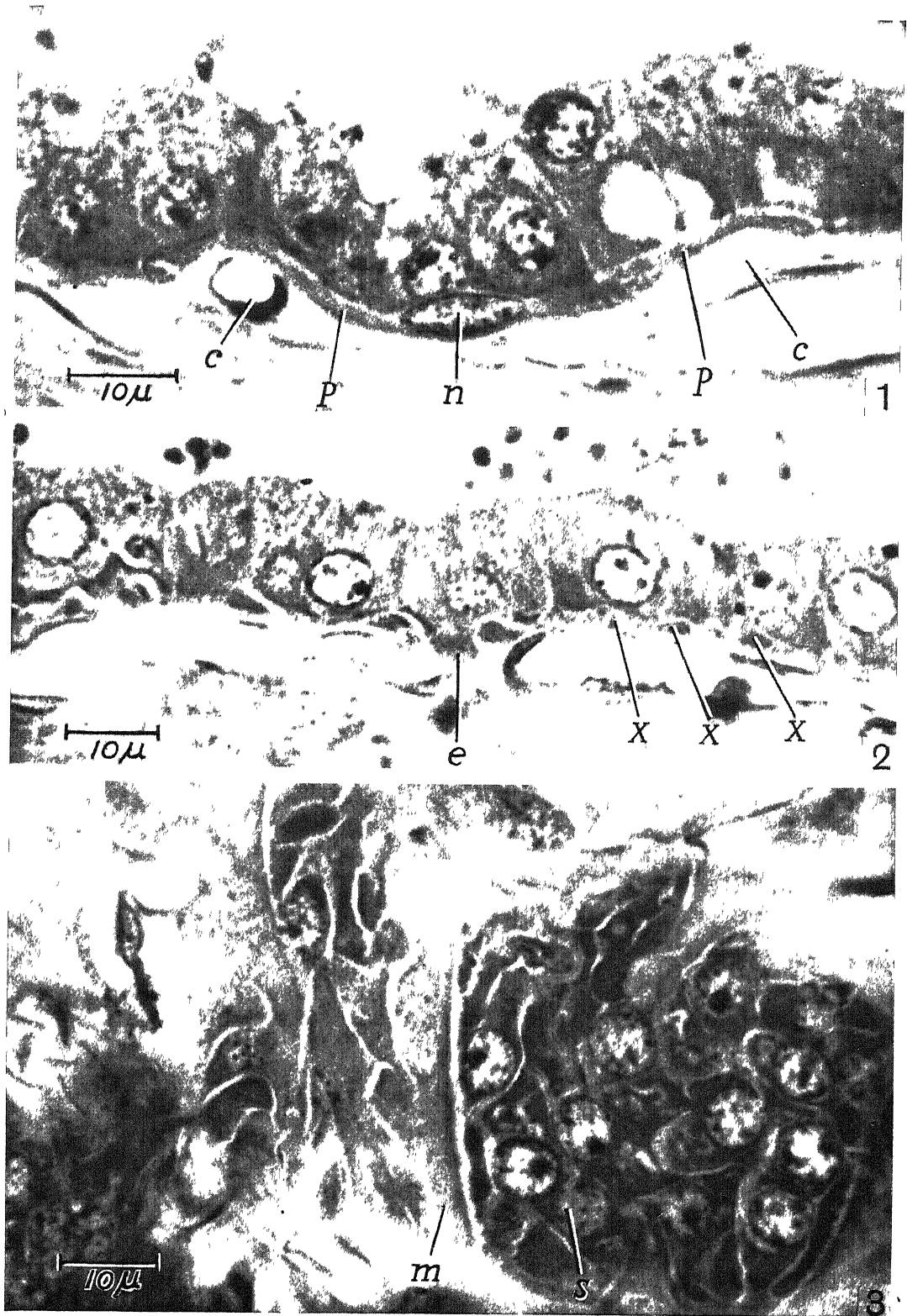
The sweat gland consists of a coiled secretory tubule, overlaid with regular longitudinal myoepithelium, and interspersed with a considerable vascular plexus. There is no smooth muscle immediately related to the tubule. The duct seems inert since it lacks smooth muscle or myoepithelium. Cannulation of a single sweat duct of the human skin with a capillary glass tube, according to Yen (1924) and Takahara (1934), shows that the secretion rises and falls as though it were being pumped in a rhythmical fashion from the gland. Buley (1938) failed to repeat the remarkable feat of cannulation but, by putting a finger in a closed chamber with adjustable humidity, he saw under the microscope an ebb and flow of sweat as it emerged from individual pores. Since sweating occurs in blanched as well as flushed skin, vascular effects are unlikely to influence the manner in which the secretion emerges from a duct. Assuming that the secretory and duct epithelia are themselves non-contractile, there are left as factors influencing the flow of secretion only the secretory response of the epithelium and a contractile activity on the part of the myoepithelium. In denervated tubules secretion can be restored by drugs like pilocarpine, but we lack information as to whether the pumping activity is also retained. Boeke (1934) has provided illustrations of supposed innervation of secretory epithelium, myoepithelium and capillary endothelium in sweat glands. Unfortunately the fibres he identifies form an unintelligible network in which it is impossible to distinguish any clear-cut pathways for separate innervation of myoepithelium. Thus there is a strong presumption that the myoepithelium of sweat tubules functions as a contractile tissue, but we are ignorant of its co-ordination and control.

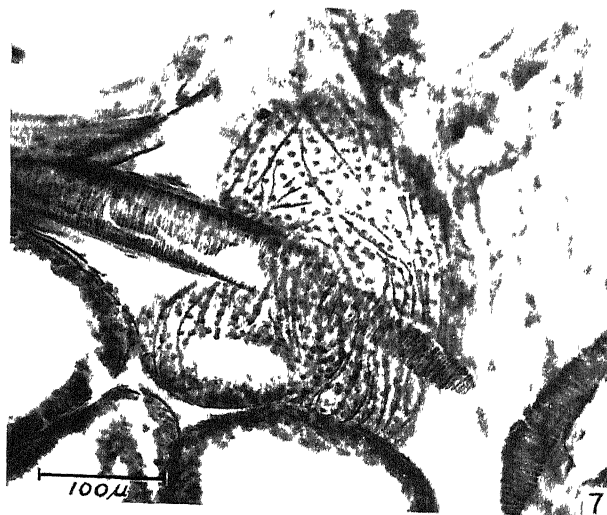
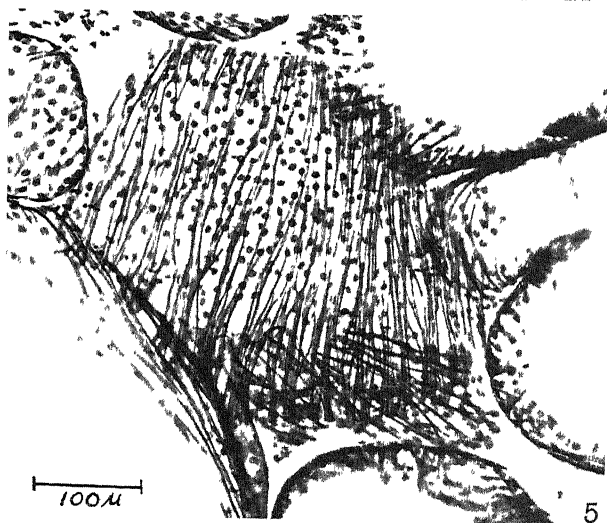
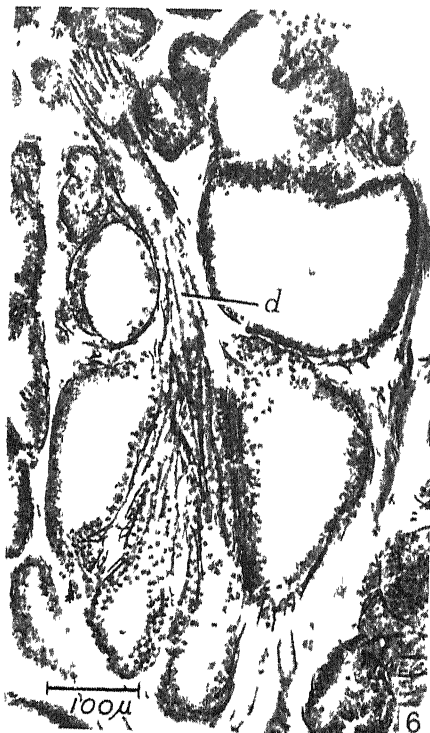
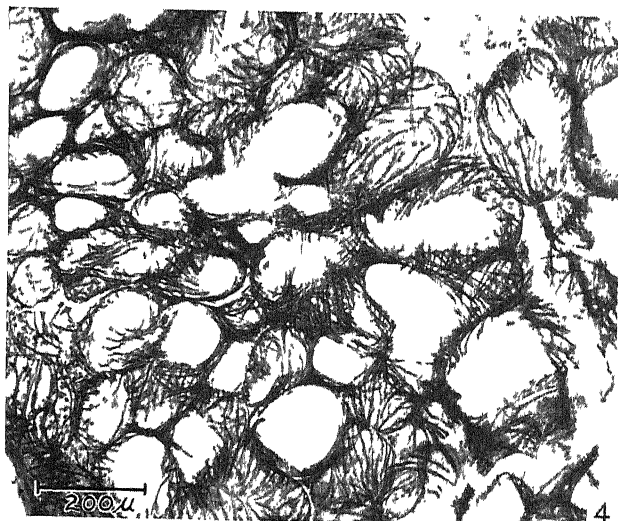
Conditions in the salivary glands are more complex and have been investigated in much greater detail. Babkin (1944) reviews the evidence which suggests that myoepithelium, or basket cells as they are called in the salivary glands, is concerned with sudden, forcible flow of saliva occurring, for instance, in the case of augmented sympathetic-after-chorda secretion. The salivary glands contain no smooth muscle apart from that associated with blood vessels, so we are dealing structurally

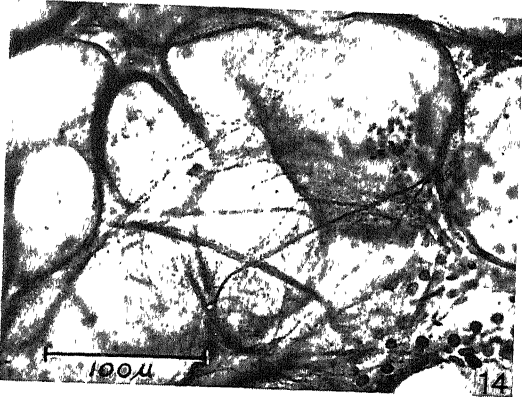
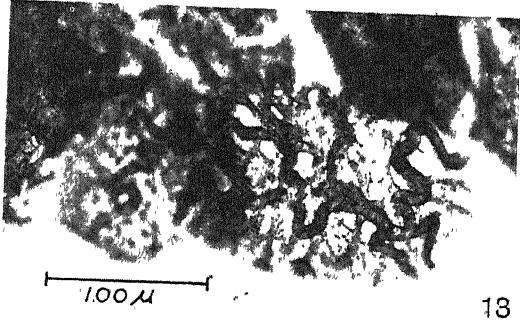
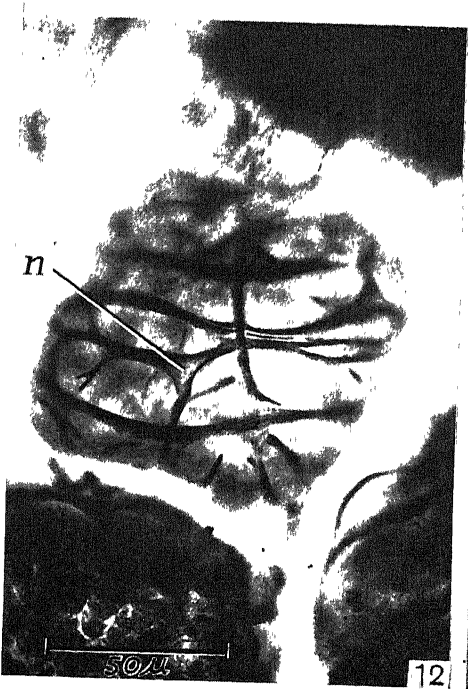
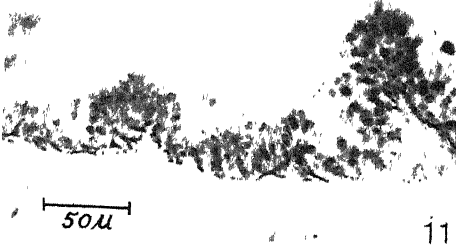
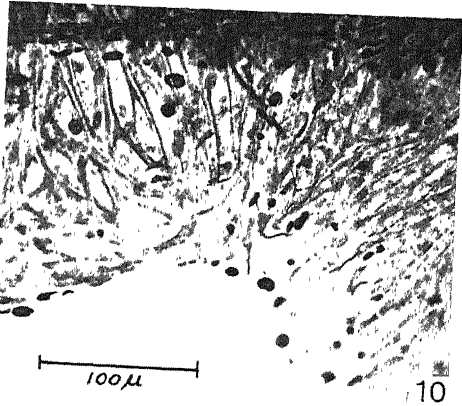
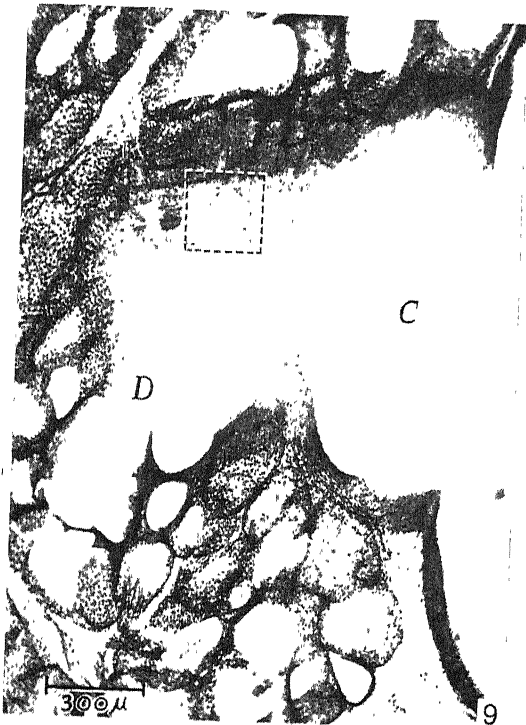
(figure 20) with secretory epithelium stimulated by secretory nerve fibres, basket cells, blood vessels in the lobules and in proximity to ducts, and feeble dilatation of some of the main ducts which may function as reservoirs. Considering these components in detail, it is largely by elimination that Babkin comes to the conclusion that augmented sympathetic-after-chorda secretion can only be explained on the basis of myoepithelial or basket cell contraction. The response is obtained after section of the cervical vago-sympathetic trunk which, it is claimed, leads to degeneration after 3 days of the vasoconstrictor and secretory fibres supplying the glands. The intact fibres must presumably end on the basket cells. Here it is necessary to assume not only that the myoepithelium is contractile, but that it is innervated by fibres distinct from those of vasomotor and secretory function, for which structural proof is lacking, though such proof would be only of incidental importance if the physiological evidence were more complete. The salivary gland basket cells are difficult to stain, and structural changes following a supposed contraction do not appear to have been looked for in appropriate experimental material. It is doubtful, of course, whether the small fluctuations in size of the salivary acinus during distension or contraction would be accompanied by any detectable change in the shape or size of the basket cells. No experimental evidence implying a contractile function for myoepithelium in the lachrymal or ceruminous glands has been found in the literature.

The tissues concerned with 'let-down' in the mammary gland, as will have been realized, are narrowed down to the inter-lobular smooth muscle bundles, the blood vessels and the myoepithelium. There are no sphincters in the duct system apart from those controlling the teat. Elastic fibres are very sparsely distributed in the lobules, except at the periphery of the gland beneath the skin, and thus elastic recoil would play an insignificant part in variations of milk-pressure. If we attempt to link up the experimental evidence concerning 'let-down' with what is known of the histology of the udder it will be seen that more information is required firstly in respect to the nerve supply. In the cow, and presumably in the goat, nerve fibres reach the udder through the ilio-inguinal, the posterior inguinal and ilio-hypogastric nerves. As far as nerve endings are concerned, the early work of Arnstein (1895), who used methylene blue, suggested that fibres of unknown origin terminated in most of the tissues of the organ, including the myoepithelium and the secretory epithelium. There is no physiological evidence that the actual secretion of milk is under nervous control. Further investigation of the efferent innervation of the mamma must be undertaken. Secondly, it would be desirable to know the exact course of blood circulation through the udder in case vascular responses play some role during 'let-down', or undergo modification when the isolated organ is perfused.

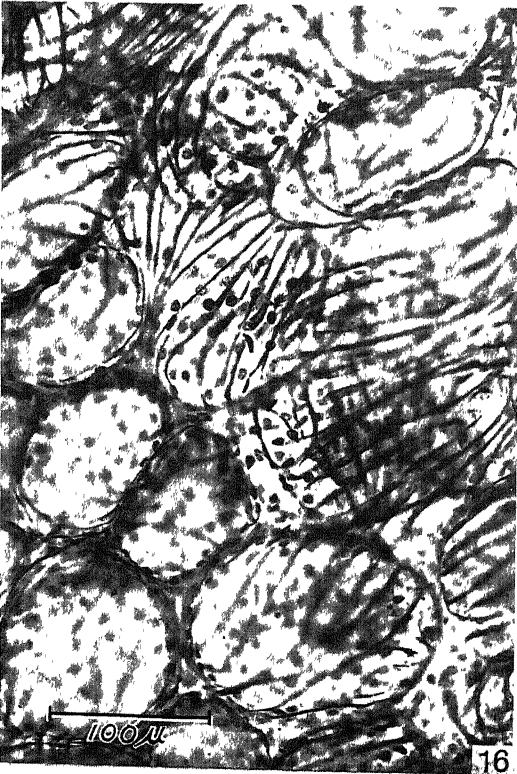
The work of Petersen and his colleagues indicates that 'let-down' is probably independent of nervous activity on the efferent side, but it leaves in doubt the full significance of the quite considerable efferent innervation of the udder, and fails to explain the function of the smooth muscle bundles scattered between the lobules. Ely & Petersen (1941) showed that 'let-down' could be evoked normally in a cow with one side of its udder denervated by cutting the ilio-inguinal and posterior inguinal nerves, but leaving the ilio-hypogastric nerve intact. Thus, on the assump-













tion that the last nerve carried only afferent impulses from the skin, they claimed to be dealing with a denervated gland in which the responses of the contractile tissue were normal. Fright, or intra-jugular injection of adrenaline inhibited 'let-down' equally on the innervated and denervated sides. Intravenous injection of pitocin or pitressin caused the denervated gland to be more completely drained than was the case with a normal milking. It was further shown by Petersen & Ludwick (1942) that the isolated udder, perfused with blood at a constant pressure, gave responses resembling 'let-down' when the blood was taken from another cow in which 'let-down' had been induced at the time of bleeding. No detailed work is recorded on the influence of variations in blood flow on milk-pressure, and it would seem essential first to establish that the circulation in an isolated perfused organ goes the same way as in the intact animal. We do not know, for instance, whether alternative vascular channels or shunts exist in the udder. Petersen (1942) tried the addition of various drugs and hormones to the perfusing fluid and showed that pitocin gave complete milk ejection from the isolated gland, at the same time reducing the blood flow by 8 to 20 %. Acetylcholine produced a complete response without affecting the blood flow. Partial ejection was obtained with substances like adrenaline and histamine which decreased the blood flow by 50 to 100 %. These experiments suggest that, if myoepithelium is to be regarded as a contractile tissue, its pharmacological responses are not the same in all the glands of the body which we have been considering.

The present work brings into clearer focus the outstanding abundance of myoepithelium in the mammary gland, and shows that its orientation is everywhere consistent with its functioning as a contractile tissue expressing milk from the alveoli and ducts. The changes in these cells, which seem to indicate that contraction does take place, are based on visual impression rather than direct measurement, because it seemed impossible to choose alveoli of comparable size and dimensions from separate distended and contracted glands. If one cannot do this, then there is no guarantee that the myoepithelial cells one measures will be comparable. They grow longer during pregnancy on the larger alveoli, and remain short and branched on the smaller ones. It is also difficult to be certain that the silver impregnation outlines each cell completely. Few cells can be traced throughout their entire length as they curve around the alveoli even in thick sections. Nevertheless the form and position of the myoepithelium in relation to the folds of contracted alveoli seem to provide as good a proof of contractile function as can be obtained from purely structural investigation. The final confirmation by direct observation of the living gland must await improvements in the methods for observing living tissues under high magnification in the intact organism.

The literature on myoepithelium in the mammary gland consists of numerous short papers which describe the appearances of these cells in thin sections. They were regarded as contractile cells similar to smooth muscle fibres by most early authors, and particularly when Bertkau (1907), using Benda's crystal violet stain for myofibrils in smooth muscle, showed that a more or less specific staining of myoepithelium could be obtained in human and dog lactating glands. This technique has fallen into disuse, being unsuitable for routine paraffin, or thick sections. It is

unnecessary to review the early literature quoted by such authors as Lenfers (1907), Retterer & Lelièvre (1911), Turner (1939) and others, because it is concerned largely with affirming the existence of a tissue which, being ectodermal in origin, was an outstanding anomaly in histogenesis. The following points seem, however, worth considering. As far as one can detect, the myoepithelium of the normal lactating gland is a discrete tissue, differentiated in form and location from smooth muscle. No areas in the present material have been found in which a transition or blending of the two cell-types occurs. Swanson & Turner (1941), studying sections stained with Van Gieson's picro-acid-fuchsin, have confirmed the existence of myoepithelium in the udder of the cow, but they vary their terminology, as indeed the title of their paper indicates, to give the impression that the term smooth muscle will suffice to describe the identity of all the contractile tissue in the gland. This is unjustified at present on the basis of our physiological knowledge of the lactating gland. It must be remembered that true smooth muscle is present in the virgin udder at maturity, if not at an earlier post-natal or even foetal stage, whereas in immature or non-pregnant animals the myoepithelium is confined to the walls of the ducts. Turner (1931), in tracing the development of the cow udder, has emphasized that the stroma is mapped out in the foetus into lobular areas of adipose tissue. The same is true for the goat (Turner & Gomez 1936). These authors suggest that the mammary ducts ramify in this fatty tissue during pregnancy and thus the original inter-lobular septa are retained as the boundaries of the glandular lobules. It is in these septa that the major blood vessels are already organized, and smooth muscle has been differentiated ahead of the glandular tissue. Whether extra smooth muscle is developed during pregnancy is still uncertain. In any case it is not found intra-lobularly. The silver-stained sections suggest that the smooth muscle bundles between the lobules have some function closely related to the blood vessels, rather than to compression of the lobules. Do these long bundles hold the lobules apart during the distension phase of secretion, and so minimize pressure on the veins and smaller vessels? It would be worth while including some reconstructions of the distribution of smooth muscle in studies on the exact pattern of the blood vessels in the lactating gland.

Myoepithelium has also been extensively studied in relation to the hyperplasias and mixed tumours of the mamma (Peyron, Corsy & Surmont 1926; Kuzma 1943; Biggs 1947), together with the mixed tumours arising in sweat, salivary (Sheldon 1941, 1943) and lachrymal glands. Such studies are, of course, pursued with an eye on the structural transformations and growth behaviour of myoepithelium as benign or malignant cells, rather than on explanation of their function in the normal gland. It is common to find, therefore, that the pale staining cells on the outer surface of the mammary duct epithelium in general are called myoepithelium, irrespective of whether they are elongated like the cells we recognize in the fully developed gland. Elongated myoepithelial cells are present on the ducts of the nipple and inter-lobular areas of the resting gland, but the distribution of similar fully differentiated cells over the rest of the duct system, which is awaiting proliferation at pregnancy, is less clearly known for individual species. It is probable that the parts of the duct system which persist unchanged from the resting phase

do not develop fresh myoepithelium; the parts which will grow extensively to form lobules develop the elongated cells as they go.

A recent paper by Dempsey, Bunting & Wislocki (1947) makes clear the need for a careful distinction between fully differentiated myoepithelial cells and the immature outer duct epithelium. These authors claim that the latter epithelium in the resting phase gives a strong reaction for the presence of alkaline phosphatase. They attempted to follow the cells with high phosphatase content through material from rats representing the growth phases of pregnancy up to full lactation. It is quite clear, however, from the illustrations and description of these authors that the phosphatase staining is spread over a broad zone of cells in the resting ducts and cannot be confined exclusively to the myoepithelium if we use this term to mean the cells similar to those found in sweat glands and the lobules of the lactating mamma. Dempsey *et al.* (1947) found it impossible to trace the phosphatase as persisting in the differentiated myoepithelium of the fully developed alveoli and finer ducts. This is not surprising in view of the poor fixation obtained with 80 % alcohol or acetone followed by paraffin imbedding. In transverse or oblique section the myoepithelial cells are far too small to be outlined sharply after indifferent fixation. These authors also comment on the presence of birefringent myofibrils in the myoepithelium of the human mamma, whereas they failed to find these fibrils in rat material. This again must depend to a certain extent upon the size of the cells and the quality of fixation. It is difficult to agree with their conclusions that in the rat the myoepithelial cells are unlikely to be contractile because they do not contain myofibrils when stained with phosphotungstic-haematoxylin, and that additional cells around the ducts, which do contain fibrils, may represent separate and distinct smooth muscle elements. Surely there is confusion on this issue between cells in various stages of differentiation. The theory suggested by these authors that myoepithelial cells may 'serve as transmitters of material from one place to another in a function analagous to that of a booster pump or a relay' is not so attractive, in the present state of our knowledge, as the assumption that these cells contract in response to the presence of oxytocin. Further work must be done under controlled experimental conditions in localizing phosphatase in the lactating gland. Folley & Greenbaum (1947), studying mammary gland phosphatase levels in the rat during pregnancy and lactation, suggest that the enzyme may be concerned with synthesis of nucleoproteins during the growth phase and possibly with casein synthesis and the capture of blood sugar molecules during lactation. In view of the known role of alkaline phosphatase in ossification it would seem worth while to study the concentration and localization of the enzyme in the resting ducts of the dog mammary gland in which hyperplasia of the myoepithelium, and the formation of mixed tumours containing cartilage or bone, is not uncommon.

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## DESCRIPTION OF PLATES 1 TO 4

## PLATE 1

The myoepithelial cell in thin sections of Zenker-formol fixed goat mamma, stained with iron haematoxylin and orange-G-erythrosin.

FIGURE 1. Alveolar epithelium showing a myoepithelial cell with a typical elongated nucleus *n* in longitudinal section. Two processes *p* of the cell extend in close contact with the secretory epithelium. Dilated capillaries *c* lie distinctly separated from the alveolar wall.

FIGURE 2. The processes of myoepithelial cells cut transversely appear as small oval or triangular bodies *X* indenting the basal epithelial membrane. In the centre, between two capillaries, an extension *e* of the secretory cell cytoplasm appears to be pinched between two myoepithelial processes.

FIGURE 3. Occasionally a tangential section through an alveolar wall is at just the right level to cut the central area of a myoepithelial cell *m*; together with the darker stained secretory epithelium *s*.

PLATE 2

The general appearance of silver-impregnated, thick sections.

FIGURE 4. Part of a lobule from a gland fixed during distension, cut at  $75\mu$ . The silver stains the myoepithelium and the nuclei of the alveolar epithelium, giving a general impression of the concentration of myoepithelium in the lactating gland.

FIGURE 5. Some alveoli, commonly found at the periphery of a lobule, grow very large. The myoepithelium is correspondingly stretched up to  $200\mu$  in length. The small round nuclei belong to the secretory epithelium.

FIGURE 6. Showing longitudinal orientation of myoepithelium on an intra-lobular duct *d* connected with some large, elongated alveoli. Section  $50\mu$  thick.

FIGURE 7. An arteriole passing across the surface of an alveolus shows smooth muscle fibres stained with silver to about the same intensity as the myoepithelium. There is some connective tissue staining in the background.

FIGURE 8. The margin of a lobule and an interlobular septum in which smooth muscle bundles *N*, coursing obliquely to the plane of section, are strongly impregnated in contrast to the connective tissue.

PLATE 3

Further details from silver-impregnated sections.

FIGURE 9. Showing the point of exit of a large duct *D* into a cistern *C* in a distended gland. Note the absence of a sphincter. The cistern epithelium within the dotted square is enlarged in figure 10.

FIGURE 10. Cistern epithelium cut tangentially showing abundant myoepithelium.

FIGURE 11. Cistern epithelium contracted, showing obliquely cut myoepithelium which is distinct from the underlying unstained connective tissue.

FIGURE 12. A small contracted alveolus in surface view, showing a myoepithelial cell with nucleus *n* and branching processes.

FIGURE 13. Blood capillaries on the walls of alveoli. Myoepithelium unstained.

FIGURE 14. Connective tissue fibres passing across the surface of alveoli. Myoepithelium unstained.

PLATE 4

The comparison between distended and contracted glands.

FIGURE 15. Part of a lobule from the left half of a goat's udder fixed while distended with milk.

FIGURE 16. Myoepithelium on the surface of distended alveoli from material comparable with figure 15.

FIGURE 17. The right half of the udder from the same goat as figure 15, which was milked out as completely as possible before fixation. Note the contracted lobules with collapsed alveoli and ducts lined with a thick folded epithelium. (Both figures 15 and 17 at the same magnification.)

FIGURE 18. Myoepithelium on contracted alveoli (cf. figure 16; both figures 16 and 18 at the same magnification).

# Some antigenic properties of mammalian spermatozoa

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[PLATES 5 TO 7]

Methods of testing sera for precipitins against seminal proteins, and for spermatozoal agglutinins are described.

The influence of various factors on agglutination of rabbit and guinea-pig spermatozoa in hanging drop preparations has been investigated. In diluted normal sera agglutination of spermatozoa by their tails occurred rarely, and then only in low serum dilutions. In the sera of immunized animals tail agglutination commenced within 10 sec., and titres could be read after 20 min. Altering the pH of the diluent below 7·0, or above 8·1, reduced the serum titres. Washed spermatozoa from semen or from the epididymis were agglutinated to higher serum titres than unwashed spermatozoa from the same specimens. A fourfold increase in the concentration of unwashed rabbit spermatozoa, and an eightfold increase in the concentration of washed rabbit spermatozoa halved the serum titre. Additions of complement did not, in the proportions used, alter the agglutination titres of antisera or the motility of the spermatozoa.

The agglutination of rabbit spermatozoa is inhibited by fluid from the vagina. It can be reversed by mechanical means, or by addition of fresh spermatozoa in excess. With guinea-pig spermatozoa disagglutination has been achieved by mechanical methods only.

Mixed agglutinates of spermatozoa of different species can be produced by antisera which agglutinate them separately.

## I. INTRODUCTION

Many different methods have been used to demonstrate and study the antigenic properties of spermatozoa. The starting point of this work was Landsteiner's observation (1899) that fresh bull spermatozoa injected intraperitoneally into normal guinea-pigs maintained activity for a considerable time; in the peritoneal cavity of guinea-pigs previously treated by a course of parenteral injections of bull spermatozoa they were rapidly immobilized.

Most subsequent authors have relied on *in vitro* tests, and immobilization of spermatozoa *in vitro* was used as a criterion of the presence of anti-spermatozoal bodies in sera by Metchnikoff (1900*a, b*), Moxter (1900), Metalnikoff (1900), Weichardt (1901), Landsteiner (1901, 1902), Kennedy (1924), Walsh (1925), and Mudd & Mudd (1929). Such results, however, must be regarded with caution, because normal fresh sera frequently exert a toxic action on spermatozoa. This was described for serum and spermatozoa of different species by Metalnikoff (1900), Weichardt (1901) and Chang (1947), and for serum and spermatozoa of the same species by Walsh (1925) and Chang (1947). Heating to 56° C for 30 min. destroyed the spermicidal activity of normal and of anti-spermatozoal sera. Metalnikoff (1900) and Weichardt (1901) found that addition of fresh serum of some species, but not of others, and in certain proportions only, would restore the toxicity to spermatozoa of heated antisera, but not of heated normal sera. It is clear that immobilization of spermatozoa is not a specific action limited to sera containing antibody.

Taylor (1908) reported the production of spermatolysins by immunizing rabbits against salmon spermatozoa. This phenomenon has not been observed with spermatozoa of other species. Alteration of the electrophoretic motility of spermatozoa was thought by Mudd & Mudd (1929) to be an adequate method of demonstrating their specific combination with antibody; but there were technical difficulties in working with spermatozoa. The complement fixation test has been used by Landsteiner & van der Schoer (1927), Henle (1938), Henle, Henle & Chambers (1938), Henle *et al.* (1940), and Parsons & Hyde (1940), to study the production and specificity of antibodies to spermatozoa.

Agglutination of spermatozoa was described by Metchnikoff (1900a), who noted that, when not instantly immobilized by antisera, rabbit spermatozoa were clumped into star-like masses. Weichardt (1901) prepared spermatozoal agglutinating and anti-agglutinating sera. McCartney (1922-23) produced agglutination of rat spermatozoa *in vitro* using the vaginal secretions of female rats previously treated by injections of rat spermatozoa or testicular extracts. None of these workers stated whether the spermatozoal heads were at the centre or the periphery of the aggregates. The agglutination of mammalian spermatozoa by their heads has since been observed in saline-diluting fluids (Chang 1947; Milovanov 1934) and in normal sera (Henle *et al.* 1938; Chang 1947).

A definite distinction between head and tail agglutination was demonstrated by Henle *et al.* (1938) in their work on the distribution of antigens in spermatozoal heads and tails, which they separated by sonic cavitation and differential centrifugation. They immunized rabbits against suspensions of whole bull spermatozoa, spermatozoal heads, and spermatozoal tails, respectively. When mixed with whole bull spermatozoa in hanging drop preparations the anti-head sera produced head agglutination, the anti-tail sera produced tail agglutination. The antisera to whole spermatozoa caused agglutination in networks and strings. After absorption with spermatozoal heads the sera prepared against whole spermatozoa produced tail agglutination, and after absorption with spermatozoal tails they produced head agglutination. Whereas tail agglutination could only be produced by the sera of immunized animals, head agglutination of spermatozoa was often seen in normal sera. The authors showed that there were head-specific and tail-specific heat-labile antigens, and also a heat-stable and species specific antigen common to both heads and tails. They carried out complement fixation tests in parallel with agglutination tests.

Agglutination of mouse spermatozoa by their tails was described by Snell & Poucher (1943) who produced iso-agglutinins. Snell (1944) depended upon agglutination to demonstrate antigenic differences between the spermatozoa of different strains of mice.

Precipitin reactions of human semen have been studied for medico-legal purposes. Injection of rabbits with human semen was shown by Dervieux (1921) and Hektoen (1922) to induce the formation of species specific precipitins for seminal proteins and extracts of spermatozoa, and for human serum. The latter could be selectively absorbed, leaving only those specific for human semen (Hektoen 1922). Landsteiner & Levine (1926) demonstrated that spermatozoa contained substances

identical with or similar to the iso-agglutinable substances *A* and *B* of human red blood corpuscles, and Hektoen & Rukinstat (1928) described a method of tracing seminal stains to persons of certain blood groups. Using serological, chemical, and electrophoretic methods, Ross (1946) showed that antisera against human seminal plasma contained precipitins in small quantities for serum albumin, and in larger amounts for seminal proteins; the latter in some respects resembled serum globulins and gave cross-precipitation with them.

The precipitation reactions of sperm-agglutinating antisera have not hitherto been reported, nor has agglutination of spermatozoa by antisera containing precipitins for seminal proteins been described. It seemed of interest to determine whether these antibodies could be produced independently of one another, and also whether the precipitin reaction always showed the high degree of species specificity found by Hektoen (1922). Using the complement fixation test, Landsteiner & van der Scheer (1927) and Henle *et al.* (1938) have shown that anti-spermatozoal sera were organ specific, but not species specific, although lower titres were obtained against heterologous spermatozoa. The technique of agglutination tests is much simpler than either precipitation or complement fixation reactions. It therefore seemed worth while to study the factors influencing the agglutination of mammalian spermatozoa *in vitro* as little information on the subject is available. The eventual object was to use this method for further studies of the antigenic relationships of spermatozoa of different species, and also to detect the presence of antibodies in the genital tract of immunized animals.

## 2. GENERAL TECHNIQUE

### *Animals used*

Antisera were prepared in rabbits, goats, and in a sheep. Semen was obtained from rabbits, dogs, goats, and bulls, by the artificial vagina technique described by Macirone & Walton (1938), and Hammond & Asdell (1926).

Epididymal spermatozoa were obtained from rabbits, goats, guinea-pigs, and rats, by killing the animals, dissecting out the vasa deferentia and epididymides, and expressing their spermatozoal contents into the diluent after making multiple incisions into the epididymides. In the case of mice, the whole epididymides and vasa deferentia were minced finely in the diluent, and subsequently the suspension was filtered through two layers of surgical gauze.

### *Preparation of spermatozoal suspensions and seminal plasma*

#### *For immunization*

Data relating to the production of antisera are summarized in table 1. For immunizing rabbits, the amount of semen, or epididymal spermatozoa, required for the whole group was collected on the day of injection. It was pooled, and diluted sufficiently to allow a dose of 2 ml. of spermatozoal suspension for each rabbit.

The rabbit seminal plasma and washed rabbit spermatozoa, used for immunizing the goats G. 1 and G. 2 respectively, were prepared from 75 samples of rabbit



TABLE 1. PREPARATION OF ANTISPERMATOZOAL SERA

species	sex	experiment number	species of spermatozoa injected	source of spermatozoa or seminal plasma	number of animals per dose	volume injected (ml.)	route of injection	number of weekly injections
goat	male	G. 1	—	rabbit seminal plasma	7½	15	I.P.	9
"	"	G. 2	rabbit	semen	7½	10	"	9
"	female	G. 3	rat	epididymis and vas	25	25	"	9
"	"	G. 4	mouse	"	250	20	"	9
"	"	G. 5	guinea-pig	"	10	15	"	8
rabbit	female	R. 8	guinea-pig	epididymis and vas	1	2	I.V.	5
"	"	R. 10						
"	"	R. 12						
"	"	R. 9	guinea-pig	epididymis and vas	1	2	inseminated P.V.	5
"	"	R. 11						
"	"	R. 13						
"	male	R. 1	rabbit	semen	1	2	I.V.	13
"	"	R. 2						
"	"	R. 3						
"	"	R. 4						
"	"	R. 5						
"	"	R. 6						
"	female	P. 1	rabbit	semen	1	2	I.V.	13
"	"	P. 2						
"	"	P. 3						
"	"	P. 4						
"	"	P. 5						
"	"	P. 6						
"	female	S. 1	rat	epididymis and vas	20	5-10	I.P.	10
sheep								

Note: I.P. = intraperitoneal; I.V. = intravenous; P.V. = per vaginam

semen collected over a period of 6 weeks, and stored frozen at  $-10^{\circ}\text{C}$ . These specimens were thawed, pooled, diluted 1 in 4 with 0.85 % NaCl, and centrifuged at about 2000 r.p.m. for 1 hr. The seminal plasma was taken off, and its volume made up to 150 ml. The deposit of spermatozoa was resuspended in 150 ml. and, after thorough mixing, centrifuged as before. The supernatant fluid was discarded, and the spermatozoa suspended in 100 ml. of fresh saline. The diluted seminal plasma and the sperm suspension were stored frozen until required for injection.

The epididymal spermatozoa of the rats, mice, and guinea-pigs, used each week for immunizing the goats G. 3, G. 4, and G. 5 respectively, were collected a few days in advance and kept frozen at  $-10^{\circ}\text{C}$  until the day of injection. The suspension of mouse spermatozoa was concentrated by centrifuging and removing some of the supernatant fluid, to reduce the volume of each dose to 20 ml.

#### *For testing the antisera*

For agglutination tests, freshly collected specimens of semen or epididymal spermatozoa were examined for motility, and diluted about 1 in 20 to give a density between  $1 \times 10^4$  and  $2.5 \times 10^4$  sperm per  $\text{mm}^3$ . With experience a suitable concentration could be judged by microscopical examination without counting.

When washed spermatozoa were required, the dilute spermatozoal suspension was centrifuged at about 2000 r.p.m. for 30 min. The dilute seminal plasma was separated, and replaced at once by the same volume of fresh diluent. After thorough but gentle mixing, the suspension was centrifuged as before, the washing fluid taken off, and the deposit of spermatozoa resuspended in fresh diluent. In some experiments the spermatozoa were washed three times, in this way, before making up the final suspension for testing.

For precipitation reactions, seminal plasma and the diluting fluid of epididymal spermatozoa, freed from spermatozoa, were used as antigens. Whole semen was diluted 1 in 5, and centrifuged at about 2000 r.p.m. for at least 1 hr. The supernatant fluid was removed immediately, leaving about 0.5 ml. above the spermatozoal deposit. It was examined microscopically for spermatozoa; if any were present it was recentrifuged. Concentrated suspensions of epididymal spermatozoa were prepared, using the epididymides of 1 guinea-pig, 3 rats, or 10 mice per ml. of diluent. They were centrifuged as described for whole semen.

#### *Diluents*

##### *For immunization*

Semen and epididymal spermatozoa for injection were diluted with 0.85 % NaCl.

##### *For testing the antisera*

In agglutination tests the buffered glucose saline solution described by Baker (1931) was the usual diluting fluid for sera and spermatozoal suspensions. To investigate the effect of pH on agglutination, solutions were made up containing 30 ml. of 10 % glucose, 30 ml. of 0.2M NaCl, 20 ml. of distilled water, and 20 ml. of buffer mixture. In the pH range 5.0 to 8.1 Sørensen's standard phosphate mixtures were used, and above pH 8.35, Auerbach & Pick's sodium carbonate-bicarbonate mixtures (Clark, 1925).

When preparing the antigen for precipitation reactions, semen and epididymal spermatozoa were diluted with 0.85 % NaCl, unless the spermatozoal deposit after centrifuging was required for agglutination tests, when Baker's solution was used. Dilutions of antiserum and further dilutions of antigen were made with 0.85 % NaCl.

#### *Route of administration*

Spermatozoal suspensions were administered to rabbits by weekly intravenous injections or by insemination into the vagina. The goats and the sheep were treated by weekly intraperitoneal injections. Details of dosage and duration of treatment are given in table 1.

#### *Collection of blood and preparation of serum*

Blood samples were obtained from all animals before a course of injections was started. During immunization samples were taken after 4 weeks, and subsequently at fortnightly intervals until bled out. A period of 4 or 5 days was allowed between the previous injection and test bleeding, and 10 days between the last injection and bleeding out. Samples were withdrawn from the marginal ear vein of the rabbit, and from the external jugular vein of the goats and sheep. The rabbits were bled out under ether anaesthesia, the goats under nembutal with chloroform-ether mixture, and the sheep under chloroform-ether mixture. The blood was allowed to clot, and the serum pipetted off and freed from red cells by centrifuging. The serum not used immediately for testing was stored at  $-10^{\circ}\text{C}$  until it could be freeze-dried. Samples of freshly separated serum were heated to  $56^{\circ}\text{C}$  for 30 min. before testing for spermatozoal agglutinins. Freeze-dried sera were reconstituted with sufficient distilled water to restore the original volume.

#### *Precipitation reactions*

##### *Ring test*

0.5 ml. of antigen was layered over an equal volume of undiluted serum in an agglutination tube. It was left at room temperature and examined after 10, 30, and 60 min. for presence of a ring at the junction of the two fluids.

##### *Flocculation test*

In order to avoid false negative reactions due to excess either of antibody or antigen, the tests were set up after the manner of Dean (1931). Undiluted antiserum and 4 dilutions (1 in 5, 1 in 10, 1 in 20, and 1 in 40) were tested against five dilutions of antigen (1 in 2, 1 in 10, 1 in 50, 1 in 250, and 1 in 1250). 0.5 ml. quantities of each antiserum and antigen dilution were mixed and observed at intervals during the first hour, at 3 hours, and at about 18 hours. The test was done qualitatively, and not with a view to determining the serum titre; therefore Dean's full range of antigen dilutions was not used, nor was the exact time of appearance of turbidity or precipitation accurately noted.

#### *Agglutination tests*

Antiserum dilutions were made, the usual range being 1 in 10, 1 in 20, 1 in 40, and so on down to 1 in 1280. 0.2 ml. of each dilution was transferred to an

agglutination tube (3 in.  $\times$  0.3 in.) and 0.2 ml. of spermatozoal suspension mixed with it. The final concentration of serum in each tube was thus halved. A control tube, containing 0.2 ml. of spermatozoal suspension mixed with an equal volume of diluting fluid, was always included.

#### *Tube agglutination*

The tubes were left to stand for 1 hr. at room temperature, and then shaken gently and examined macroscopically. The following degrees of agglutination were recognized:

- + Fine floccules just appreciable to the naked eye, or with a hand lens, suspended in a turbid fluid.
- + + Medium sized floccules suspended in an opalescent or turbid fluid.
- + + + A single pellet, or large flocculent masses, suspended in a clear surrounding fluid.

The titre of the serum was given as the reciprocal of highest final dilution in which macroscopical agglutination of the one-plus degree was seen. To exclude a false positive due to non-specific head agglutination, a drop was removed from this tube for microscopical examination.

#### *Slide agglutination*

Suspensions of active spermatozoa were used. Immediately after mixing with the diluted serum, a drop was withdrawn and made into a hanging drop preparation, which was examined microscopically after 20 min. at room temperature. Three degrees of agglutination were recognized, and recorded as follows:

- + Small rosettes or clusters of spermatozoa agglutinated by their tails were seen, under the low power, widely separated by large numbers of free spermatozoa.
- + + The majority of spermatozoa were agglutinated by their tails in loose rosettes or clusters of moderate size, a few freely-swimming un-agglutinated spermatozoa being present.
- + + + All the spermatozoa were agglutinated by their tails in dense rosettes or clusters, with their heads free on the periphery and lashing vigorously.

The titre of the serum was recorded as the reciprocal of the highest final dilution giving a one-plus degree of agglutination. When a few spermatozoa were agglutinated by their tails, in groups so small as to be identifiable only under the high power, it was recorded as a 'trace'.

Head agglutination (figure 1, plate 5) was regarded as a non-specific reaction. It was frequently seen in fresh suspensions of rabbit and guinea-pig spermatozoa in 0.85 % NaCl, or after several hours in Baker's solution. It was particularly common with the epididymal spermatozoa of guinea-pigs, and with rabbit spermatozoa suspended in diluents at pH's higher than 7.8.

*Photomicrographic technique*

The photomicrographs of figures 1 to 7, plates 5 to 7 were obtained with the phase-contrast microscope, after the method of Zernike (1942*a* and *b*). A drop of the suspension to be photographed was placed on a slide and exposed to osmic acid vapour for 30 sec. A coverslip was then sealed on with vaseline-paraffin mixture. It is a pleasure to acknowledge the assistance of Mr Smiles and Mr Welch, who adapted phase microscopy for the examination of spermatozoa, and to whom I am indebted for the following note:

‘By the usual method of direct illumination, the images of unstained spermatozoa appear as black outline pictures on a bright background. When visibility is improved by reducing the effective working aperture of the objective, undesirable diffraction fringes appear.

‘By the phase-contrast method, unstained spermatozoa appear as solid bodies, with a dark head and tapering filamentous tail. No diffraction fringes are seen, but the heads are surrounded by a bright halo, due to the differences between the refractive indices of the spermatozoa and of the suspending fluid. Satisfactory photomicrographs with minimal contrast between the halos and the spermatozoa are obtained by using panchromatic plates (Ilford HP 3).

‘The microscope and positive phase-contrast equipment used were manufactured by Messrs. Cooke, Troughton, & Sims Ltd.’

### 3. COMPARISON OF *IN VITRO* TESTS

*Precipitation tests*

The ring test is quicker and more convenient to perform than the flocculation test. Unfortunately, freshly separated sera, from normal or injected rabbits, gave well marked rings in control tests with Baker's solution, and with phosphate buffer solutions. Some normal sera gave rings with seminal plasma diluted with 0.85 % NaCl. In contrast, freeze-dried sera did not appear to give these non-specific reactions in ring tests (see table 2). In flocculation tests with fresh sera the control tubes showed a faint opalescence appearing at once. This was easily distinguishable from the true precipitation occurring with antiserum and antigen mixed in proportions approaching the optimum. Furthermore, positive reactions were obtained in the flocculation test with several sera which had given negative results by the ring test, e.g. the goat sera G. 1 to G. 4, and rabbit seminal plasma (see table 3). Because most of the sera under investigation were freshly separated, and for its greater accuracy and specificity, the flocculation test was generally preferred.

*Agglutination tests*

The same titre was usually obtained when agglutination tests were done by the tube and slide methods with the same antiserum and spermatozoal suspension (table 4). Sometimes immotile or very sluggishly motile spermatozoa, such as those from the rat epididymis, would agglutinate by the tube technique when they failed to do so in a hanging drop. The aggregates so produced were, however, of

TABLE 2. SUMMARY OF RING TESTS

	control tests				antigens			
	Baker's solution	phosphate buffer pH 7.8	3 % glucose + 0.2 % NaCl	0.85 % NaCl	rabbit seminal plasma diluted 1 in 10 with 0.85 % NaCl	dog seminal plasma diluted 1 in 10 with 0.85 % NaCl	bull seminal plasma* (a)	0.85 % NaCl suspending fluid from guinea-pig epididymal sperm (b)
A. fresh rabbit sera								
4 normal rabbit sera								
R. 5 to 10	+	+	0	0	+ weak	.	+	0
P. 1 to 6	+	+	0	0	+ weak	.	+	0
	+	+	0	0	+ weak	.	+	0
B. freeze-dried rabbit sera								
2 normal rabbit sera	0	0	0	0	0	.	0	0
R. 8	0	0	0	0	0	+	0	0
R. 12	0	0	0	0	0	0	0	0
C. freeze-dried sheep serum								
S. 1	0	0	0	0	+	+	0	0
D. freeze-dried goat sera								
normal goat sera								
G. 1	0	.	.	0	.	.	.	.
G. 2	0	.	.	0	0	.	.	.
G. 3	0	.	.	0	+	.	.	.
G. 4	0	.	.	0	0	.	.	.
	0	.	.	0	+	.	.	.

\* (a) Diluted 1 in 10 with Baker's solution.

(b) Diluted 1 in 10 with 0.85 % NaCl.

TABLE 3. SUMMARY OF FLOCCULATION TESTS

sera	antigens								controls	
	dilute seminal plasma of semen from				suspending fluid of epididymal sperm of				0.85% NaCl solution	Baker's solution
	rabbit	bull	dog		guinea-pig	rat	mouse	goat		
A. fresh rabbit sera										
normal rabbit serum	0	.	.		0	.	.	.	0	0
R. 5 to 10	0	.	.		0	.	.	.	0	0
P. 1 to 6	0	.	.		0	.	.	.	0	0
B. freeze-dried rabbit sera										
normal rabbit serum	0	.	.		0	.	.	.	0	0
R. 8	0	.	.		+	.	.	.	0	0
R. 12	0	.	.		+	.	.	.	0	0
C. freeze-dried sheep serum										
S. 1	+	0	.		+	+	.	.	0	0
D. fresh goat sera										
normal goat serum	0	0	0		0	0	0	0	0	0
G. 1	+	+	doubtful		+	+	+	0	0	.
G. 2	+	+	doubtful		+	+	+	0	0	.
G. 3	+	+	.		+	+	.	0	0	.
G. 4	+	+	.		+	.	+	0	0	.

a different nature from those obtained with motile spermatozoa, and consisted of an irregular, loose entanglement of spermatozoa.

The advantage of the tube method was a great economy of time by eliminating the preparation and scrutiny of numerous hanging drops. Its chief disadvantage was the difficulty of reading the end point. Non-specific head agglutination was easily mistaken for a one-plus degree of tail agglutination, even with the control

TABLE 4. COMPARISON OF SPERM AGGLUTININ TITRES OF ANTISERA TESTED BY THE TUBE AND SLIDE METHODS

antiserum	species of spermatozoa					
	guinea-pig		rabbit		rat	
	tube	slide	tube	slide	tube	slide
R. 8	400	400	0	0	.	.
R. 9	0	0	0	0	.	.
R. 10	20	50	20	20	.	.
R. 11	0	0	0	0	.	.
R. 12	100	200	0	0	.	.
R. 13	0	0	0	0	.	.
S. 1	400	200	100	100	2560	0
G. 1	0	0	0	0	.	.
G. 2	640	640	2560	1280	.	.
G. 3	640	640	640	640	1280	0
G. 4	320	320	640	320	.	.
G. 5	.	640	320	320	.	.

tube for comparison; or if there was much head agglutination in the control tube a one-plus degree of agglutination could be missed. Moreover, the tube method gave no indication of the motility and behaviour of the spermatozoa in different sera, or under different conditions of temperature, pH, and salt content. The slide technique appeared to present the advantages of greater specificity, accuracy, and interest, and the factors affecting slide agglutination were therefore further investigated.

*Results by precipitation and agglutination tests*

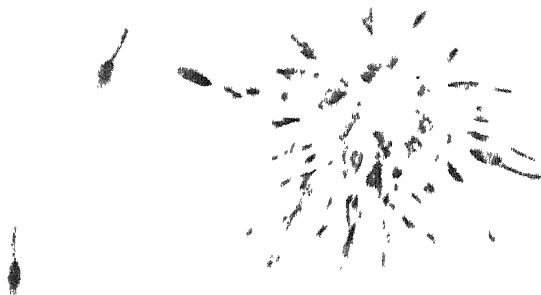
The antiserum G. 1 prepared against rabbit seminal plasma failed to agglutinate the spermatozoa of guinea-pigs, rabbits, and rats (table 4). The same serum gave positive precipitin tests with dilute seminal plasma of rabbits, and with the suspending fluid of epididymal spermatozoa from guinea-pigs and rats (table 3). The presence in sera of agglutinins for spermatozoa in the absence of precipitins for seminal proteins of the same species has not so far been demonstrated.

4. THE EFFECT OF NORMAL SERA ON THE MOTILITY AND AGGLUTINATION OF SPERMATOZOA

*Motility*

Chang (1947) showed that the spermicidal factor in fresh sera from rabbit, guinea-pig, rat, bull, and human, was unstable, thermolabile, and had several characteristics similar to those of complement.





1



2

3

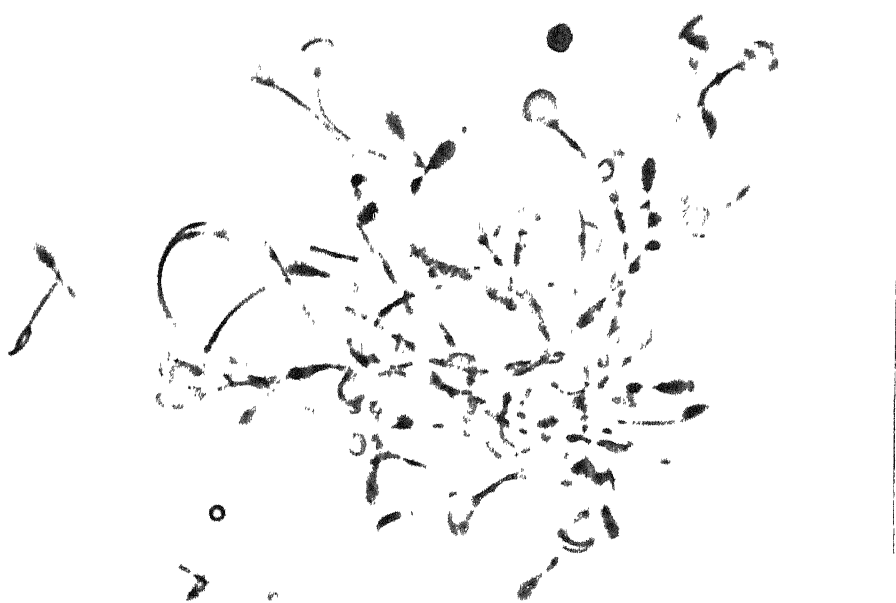


4



5

6



7



The spermicidal action of fresh rabbit, guinea-pig, and human sera, on rabbit and guinea-pig spermatozoa was confirmed using hanging drop preparations. Fresh goat serum was only weakly spermicidal. The effect was abolished by heating to 55° C for 30 min.

It was found, in addition, that sera of normal goats, rabbits, and guinea-pigs, which had been freeze-dried and reconstituted, without heating at any stage, would support the full motility of rabbit and guinea-pig spermatozoa up to 60 min. This was true even in the case of guinea-pig sera freeze-dried to preserve complement, and which, in dilutions of 1 in 33 and 1 in 50 respectively, were found to haemolyze an equal volume of 2 % sheep cells maximally sensitized with haemolysin by the method of Wadsworth, Maltener & Maltener (1938). Clearly in these two sera the spermicidal factor was not identical with the complement for the haemolytic system.

#### *Slide agglutination*

In normal sera, and dilutions thereof, agglutination of rabbit and guinea-pig spermatozoa by their heads was commonly seen. Tail agglutination, on the other hand, was rare, and then occurred only in high serum concentrations. Several samples of normal human serum produced typical tail agglutination of rabbit spermatozoa to a titre of 10, one sample only had a titre as high as 50. Normal goat serum caused tail agglutination of rabbit and goat spermatozoa to a titre of 20. In normal rabbit and guinea-pig sera, tail agglutination of spermatozoa of those species was not seen.

With sera of immunized animals, only titres for tail agglutination of 40 or over were regarded as significant. Below that serum concentration it appeared, in contrast to head agglutination, to be a highly specific phenomenon.

### 5. FACTORS INFLUENCING THE SLIDE AGGLUTINATION TEST

#### *Time*

Agglutination of spermatozoa in high concentrations of potent antisera began within 10 sec., and was advancing rapidly by 30 sec. At 2 min. the majority of spermatozoa were agglutinated by their tails in small clusters (figure 2, plate 5), and between 2 and 5 min. the small clusters coalesced (figure 3, plate 5). Although the rosettes and clusters continued to increase in size, an alteration in the degree of agglutination was seldom recorded in high serum concentrations after 5 min. (figures 4 and 5, plate 6). In low serum concentrations there was often a change between 5 and 10 min., but there was not usually an increase in titre between 10 min. and 2 hr. Typical results are shown in table 5.

#### *Temperature*

With rabbit and guinea-pig spermatozoa there was no difference between the serum titres recorded at room temperature, and those obtained when working at 37° C. Nor was there an appreciable alteration in the rate of agglutination during the first 20 min. at 37° C.

TABLE 5. THE INFLUENCE OF TIME ON THE AGGLUTINATION OF RABBIT SPERMATOZOA BY ANTISERUM IN HANGING DROP PREPARATIONS

dilutions of antiserum S. 1	time after mixing						
	30 sec.	1 min.	2 min.	5 min.	10 min.	30 min.	120 min.
1 in 10	trace	+	++	+++	+++	+++	+++
1 in 20	trace	+	++	+++	+++	+++	+++
1 in 50	trace	trace	+	++	++	++	++
1 in 100	0	trace	trace	+	+	+	+
1 in 200	0	0	trace	trace	+	+	+
1 in 400	0	0	0	0	0	0	trace

*Comparison of normal (0.85 %) sodium chloride and Baker's solution  
as the diluting fluid*

When testing samples of the same serum against freshly diluted spermatozoa from the same specimen, agglutination at slightly higher titres was usually observed with 0.85 % sodium chloride as the diluent than with Baker's solution. In saline the motility of the spermatozoa fell off rapidly, head agglutination occurred, and lower serum titres were obtained using saline suspensions more than 2 hr. old. For routine use as a diluent Baker's solution was therefore preferred.

*pH of the diluent*

Glucose saline solutions buffered at different pH's were prepared according to the prescriptions given in table 2. A number of freshly collected specimens of rabbit semen were mixed and diluted 1 in 20 with 0.85 % NaCl. Samples were withdrawn and centrifuged to separate the dilute seminal plasma. The deposits were diluted to the same final concentration of spermatozoa, using for each a solution of different pH. A series of antiserum dilutions made with the same buffer solution was used to test each spermatozoal suspension.

Results are given in table 6, and show that between pH 7.0 and 8.1 there was little difference in the serum titres recorded or in the motility of the spermatozoa observed at 20 min. Above pH 8.1 lower serum titres were obtained, and head agglutination became increasingly prominent. Below pH 7.0 the serum titre was sometimes reduced and the motility of the spermatozoa diminished. Both head agglutination and alterations in motility were most conspicuous in low serum concentrations, and in controls. Except at the extremes of the pH range the final pH of the mixtures of diluted serum and spermatozoal suspension differed little from those of the diluent used.

*Washing the spermatozoa*

Rabbit spermatozoa from diluted semen were washed free from seminal plasma, and suspended in fresh Baker's solution as described on p. 50. They were agglutinated by higher dilutions of antiserum than the same concentration of unwashed spermatozoa in the original diluted semen. When the washed spermatozoa were separated,

and resuspended in dilute seminal plasma they behaved like the original suspension of unwashed spermatozoa. The effect of washing rabbit epididymal spermatozoa was even more striking (table 7).

TABLE 6. THE INFLUENCE OF pH ON AGGLUTINATION AND MOTILITY OF RABBIT SPERMATOZOA

anti-serum	observations at 20 min.	pH of the diluent										
		5.0	5.2	5.5	6.0	6.8	7.0	7.3	7.5	8.1	8.6	9.75
S. 1	serum titre	.	.	80	320	.	320	.	320	.	160	80
	motility of sperm	.	.	trace	+	.	+++	.	+++	.	+++	+++
	head agglutination	.	.	0	0	.	0	.	+	.	+	+++
G. 2	serum titre	640	.	.	640	.	640	1280	1280	1280	640	640
	motility of sperm	trace	.	.	trace	.	+	++	++	++	+	+
	head agglutination	0	.	.	0	.	0	0	0	+	+	+
G. 4	serum titre	.	320	.	320	320	.	320	320	320	160	160
	motility of sperm	.	trace	.	trace	++	.	++	+++	+++	++	++
	head agglutination	.	trace	.	+	+	.	+	++	++	++	++
pH of the final mixture of diluted serum and sperm suspension		.	5.7	.	6.2	6.8	.	7.3	7.5	8.1	8.6	9.6

TABLE 7. THE EFFECT OF WASHING ON THE AGGLUTINATION OF RABBIT SPERMATOZOA BY ANTISERUM

final dilution of antiserum	agglutination in suspensions of					
	rabbit spermatozoa from semen			rabbit epididymal spermatozoa		
	A.	T.W.	R.S.	A.	T.W.	R.S.
S. 1						
1 in 10	+++	+++	+++	+++	+++	+++
1 in 20	+++	+++	+++	++	+++	++
1 in 50	++	+++	++	0	+++	0
1 in 100	+	+++	+	0	+++	0
1 in 200	0	++	0	0	+++	0
1 in 400	0	++	0	0	++	0
1 in 800	0	0	0	0	0	0

Note: A. Original suspension of rabbit spermatozoa in Baker's solution. T.W. Suspension of thrice washed rabbit spermatozoa in fresh Baker's solution. R.S. Suspension of thrice washed rabbit spermatozoa in dilute seminal plasma.

Suspensions of washed epididymal spermatozoa from guinea-pigs were also agglutinated to higher serum titres than unwashed spermatozoa from the same source. When the washed spermatozoa were resuspended in the original diluent the titre was reduced (table 8). When they were resuspended in dilute rabbit seminal plasma there was a reduction of titre of the antiserum S. 1 which agglutinates both rabbit and guinea-pig spermatozoa, but not of the antiserum R. 8 which only

agglutinates guinea-pig spermatozoa (tables 4 and 8). The marked difference between titres obtained with washed and unwashed spermatozoa suggested that antigens present on the spermatozoa might also be present in soluble form in the seminal fluid. To increase the sensitivity of the slide agglutination test suspensions of washed spermatozoa should be used.

TABLE 8. THE EFFECT OF WASHING, AND OF RABBIT SEMINAL PLASMA, ON THE AGGLUTINATION OF GUINEA-PIG EPIDIDYMAL SPERMATOZOA

final dilution of antiserum	agglutination in suspensions of guinea-pig epididymal spermatozoa				
	U.	T.W.	R.S.	U.S.P.	T.W.S.P.
S. 1: 1 in 40	+++	+++	+++	++	+++
1 in 80	+++	+++	++	+	++
1 in 160	++	+++	+	0	++
1 in 320	+	++	0	0	+
1 in 640	0	++	0	0	0
1 in 1280	0	trace	0	0	0
R. 8: 1 in 40	+++	+++	+++	+++	+++
1 in 80	+++	+++	+++	+++	+++
1 in 160	++	+++	++	++	+++
1 in 320	+	++	+	+	++
1 in 640	0	++	0	0	++
1 in 1280	0	+	0	0	+

*Note:* U. Original suspension of unwashed spermatozoa in Baker's solution. T.W. Suspension of thrice washed spermatozoa in Baker's solution. R.S. Suspension of thrice washed spermatozoa in supernatant fluid removed before washing. U.S.P. Suspension of unwashed spermatozoa in rabbit seminal plasma. T.W.S.P. Suspension of thrice washed spermatozoa in rabbit seminal plasma.

#### *Different samples of spermatozoa*

The same titre was not always obtained when an antiserum was tested against different diluted specimens of rabbit semen on the same day, or on successive days. Specimens with poor motility, or containing much granular debris, tended to be agglutinated to lower titres. Sometimes there was no obvious abnormality of the semen to account for poor agglutination. When the spermatozoa from different specimens of semen were washed, there was less variation in titre. Suspensions of epididymal spermatozoa from different guinea-pigs gave consistent results, unless there was much head agglutination or exceptionally poor motility. Because of the uncontrollable variation between samples of semen, antisera should always be tested against specimens from several different animals.

#### *Relation of serum titre to spermatozoal concentration*

Two specimens of highly active rabbit semen were mixed. One half was withdrawn, and dilutions of 1 in 5, 1 in 10, 1 in 20, 1 in 40, 1 in 80, and 1 in 160, were prepared with Baker's solution as diluent. The other half was diluted 1 in 20 with Baker's solution, and distributed into six centrifuge tubes. The dilute seminal plasma was separated by centrifuging, and the spermatozoal deposit in each tube washed twice.



Baker's solution was added to give concentrations of washed spermatozoa in the different tubes corresponding to dilutions of 1 in 5, 1 in 10, etc. of whole semen. The titre of the antiserum S. 1 was tested against each of the suspensions of unwashed and washed spermatozoa. The results given in table 9 show that a fourfold increase in the concentration of unwashed rabbit spermatozoa in diluted semen halved the serum titre, while with washed spermatozoa an eightfold increase in concentration was required to halve the titre.

TABLE 9. THE EFFECT OF THE CONCENTRATION OF SPERMATOZOA ON SERUM TITRE

spermatozoal suspension corresponding to semen dilution of	titre of serum S. 1	
	unwashed	washed
1 in 5	40	320
1 in 10	80	320
1 in 20	80	320
1 in 40	160	640
1 in 80	160	640
1 in 160	320	640

*The effect of added complement on motility and agglutination in antisera*

The agglutination and motility of rabbit and guinea-pig spermatozoa in antisera with and without added complement was compared. The source of complement was freeze-dried guinea-pig serum. This, when reconstituted and diluted 1 in 50, would haemolyze an equal volume of 2 % sheep cells maximally sensitized with haemolysin.

Results are given in table 10, and show that addition of complement in the proportions used was usually without effect on the serum titre or on the motility of the spermatozoa in the slide agglutination test.

Metchnikoff (1900) and Weichardt (1901) found that, in order to immobilize the spermatozoa, fresh guinea-pig serum had to be added to decomplemented antiserum in the proportion of 15 or 20 vol. to 1 vol. It is possible that, in the tests described above, insufficient complement was added to affect the motility of the spermatozoa.

TABLE 10. THE EFFECT OF ADDED COMPLEMENT ON ANTISERUM TITRE AND SPERMATOZOAL MOTILITY

a. suspensions of rabbit spermatozoa										control containing no antiserum complement dilution	
final dilution of complement											
anti-serum	no complement		1 in 10		1 in 20		1 in 40		1 in 10		
	titre	motility	titre	motility	titre	motility	titre	motility	titre	motility	
S. 1	320	+++	320	+++	320	+++	320	+++	0	+++	
G. 2	1280	+++	640	0	1280	+	1280	+++	0	0	
G. 3	320	++	160	++	160	++	320	++	0	++	
G. 4	320	+++	320	++	320	++	320	++	0	++	
b. suspensions of guinea-pig spermatozoa											
S. 1	320	+++	320	+++	320	+++	320	+++	.	.	
G. 2	640	+++	640	+++	640	+++	640	+++	.	.	

## 6. INHIBITION AND REVERSAL OF AGGLUTINATION

*Inhibition of agglutination*

The vaginae of several living rabbits were douched, each with about 5 ml. of Baker's solution introduced with a syringe through a rubber catheter and run in and out several times. The fluid recovered was faintly opalescent, and contained small numbers of squamous epithelial cells. Washed rabbit spermatozoa suspended in the vaginal douchings were agglutinated to low titres, or not at all, by potent antiserum. When these suspensions were centrifuged and the deposits of spermatozoa resuspended in fresh Baker's solution, the agglutination titre was the same as for the control suspension of washed spermatozoa. Table 11 gives the results of these experiments.

TABLE 11. THE EFFECT OF VAGINAL DOUCHING FLUID ON THE AGGLUTINATION OF RABBIT SPERMATOZOA

experiment no.	titre of antiserum S. 1			
	I	II	III	IV
	unwashed spermatozoa	washed spermatozoa in Baker's solution	washed spermatozoa in douching fluid	spermatozoa from III resuspended in fresh Baker's solution
1	80	320	no agglutination	
2	160	320	40	320
3	160	320	40	320
4	160	320	80	320
5	160	640	no agglutination	640

The following substances when added to Baker's solution in the concentrations stated failed to influence the agglutination titres: 0.25 % soluble starch; 0.25 % gelatin; 0.25 % gum arabic, 0.25 % sodium silicate; 1 in 10 normal freeze-dried rabbit serum.

*Reversal of agglutination**By mechanical means*

Spermatozoa which had been exposed for short periods to antiserum, and had been agglutinated thereby, could be dissociated by shaking the agglutination tube or by bubbling air through the mixture. If then allowed to stand undisturbed they would re-agglutinate to the same degree as before. If, after disagglutination, the spermatozoa were washed till the supernatant fluid was free from all traces of serum, and resuspended in fresh Baker's solution, they would re-agglutinate in the absence of added serum. This suggested that a firm union between antigen molecules on the tails of the spermatozoa and antibody from the serum had taken place, and that the agglutination was a separate process. Experiments are described below.

*Experiment 1.* A suspension of guinea-pig epididymal spermatozoa was mixed in an agglutination tube with an equal quantity of a 1 in 50 dilution of the anti-

serum R. 8. After standing for 5 min. the three-plus degree of agglutination had been reached. By bubbling air through the suspension the spermatozoa were dissociated, and no agglutination was seen macroscopically or microscopically. When allowed to stand undisturbed for 5 min. the spermatozoa re-agglutinated to the three-plus degree.

When the initial exposure to serum was 5, 10, 20, or 40 min. the agglutinated spermatozoa could be dissociated by air bubbles or by shaking. After 60 min. exposure to serum treatment they could not be dissociated.

*Experiment 2.* 1 ml. of the suspension of guinea-pig epididymal spermatozoa, and 1 ml. of the 1 in 50 dilution of the antiserum R. 8, were mixed and allowed to stand for 20 min. Agglutination (three-plus) had occurred. 8 ml. of Baker's solution was added to make the final serum concentration 1 in 500; the titre of the serum was 320. The suspension was well mixed and no agglutination was seen macroscopically or microscopically. It was centrifuged, the supernatant fluid removed, and 2 ml. of fresh Baker's solution added to resuspend the deposit. On immediate examination no agglutination of spermatozoa in this suspension was seen, but after standing for 10 min. they were agglutinated to the three-plus degree. Another 8 ml. of Baker's solution was added and centrifuging, resuspending, and examinations carried out as before, with the same result. After a third washing, using the same technique, the spermatozoa still re-agglutinated when allowed to stand, although no trace of serum could have been present in the suspending fluid. The first, second, and third supernatant fluids all failed to agglutinate fresh guinea-pig spermatozoa not previously treated with serum.

These experiments were repeated using higher dilutions of the same antiserum R. 8, and with the antiserum S. 1 in various dilutions tested against rabbit and guinea-pig spermatozoa. In every case similar results were obtained.

#### *Addition of an excess of fresh spermatozoa*

When an excess of fresh rabbit spermatozoa was added to serum-treated spermatozoa of the same species, re-agglutination, which would otherwise follow mechanical separation, could be prevented, except when the serum treatment had lasted 16 min. or longer. A typical experiment is described below.

The titre of the antiserum was determined with different concentrations of rabbit spermatozoa. With a 1 in 16 dilution of semen the titre of the antiserum S. 1 was 1 in 320; with a 1 in 4 dilution of the same semen it was 1 in 160. Equal quantities of the 1 in 320 dilution of S. 1 and the 1 in 16 dilution of semen were measured into a beaker. At 1, 2, 4, 8, 16, and 32 min. thereafter the contents of the beaker were thoroughly remixed, and 1 ml. transferred to a tube containing the centrifuged deposit from 4 ml. of the 1 in 16 semen dilution. There was thus an eightfold excess of fresh spermatozoa, not previously treated by serum, in contact with the serum-treated spermatozoa. Immediately after mixing the contents of the tube a drop was examined under the microscope. In no tube was any agglutination seen at this time. 20 min. later another drop was removed for examination. In the tubes containing spermatozoa which had been treated with serum for 16 and 32 min. prior to adding fresh spermatozoa, agglutination of the one-plus degree was seen.

In the tubes in which the preliminary serum treatment had lasted 1, 2, 4, and 8 min. respectively, no agglutination was seen.

This suggested that, when serum treatment had lasted 8 min. or less, the original rabbit spermatozoa shared antibody with the excess of fresh spermatozoa, and did not re-agglutinate because on no sperm tail was sufficient antigen combined with antibody to enable it to adhere firmly to others.

It appeared as though, after 16 min. exposure to serum, the original rabbit spermatozoa would not part with antibody to the fresh spermatozoa, and therefore re-agglutinated to the one-plus degree.

When an excess of fresh guinea-pig spermatozoa was added to serum-treated spermatozoa of the same species, re-agglutination was not prevented, even when the spermatozoa and serum had only been in contact for 15 sec.

## 7. MIXED AGGLUTINATION OF SPERMATOZOA FROM DIFFERENT SPECIES

Some of the antisera would agglutinate to high titres spermatozoa from several different species. When tested against suspensions containing mixtures of spermatozoa from those species, mixed agglutination occurred, the rosettes being composed of the different types of spermatozoa agglutinated together by their tails. The spermatozoa of rabbit, guinea-pig, and mouse, are particularly easy to distinguish by their characteristic heads; these were free at the periphery of the rosettes, leaving no doubt as to the phenomenon of mixed agglutination (figures 6 and 7, plate 7). Some experiments are described below.

*Experiment 1.* A suspension containing rabbit and guinea-pig spermatozoa was treated for 15 min. with an equal volume of antiserum S. 1 diluted 1 in 25. A 3 plus degree of agglutination with rosettes composed of spermatozoa of the two species mixed was produced. These agglutinates were broken up by mechanical means and the spermatozoa washed three times as previously described. When resuspended, and allowed to stand undisturbed, mixed rosettes reformed.

*Experiment 2.* When fresh rabbit spermatozoa were added to serum-treated, disagglutinated, and washed guinea-pig spermatozoa, agglutination (3 plus) of guinea-pig spermatozoa occurred, but no agglutination of rabbit spermatozoa. A possible explanation was that the guinea-pig spermatozoa would not share antibodies with rabbit spermatozoa.

*Experiment 3.* When fresh guinea-pig spermatozoa were added to serum-treated, disagglutinated, and washed rabbit spermatozoa there was an early stage in which rosettes of agglutinated rabbit spermatozoa reappeared. Later, all these rosettes had disappeared, and there was no agglutination of either rabbit or guinea-pig spermatozoa. This suggested that the rabbit spermatozoa had parted with antibodies in presence of guinea-pig spermatozoa.

Some features of the antigen-antibody reaction of spermatozoa are of general serological interest. The formation of mixed aggregates of spermatozoa of different species in presence of antiserum is not in accordance with the lattice hypothesis put forward by Marrack (1934) to explain the phenomena of precipitation and agglutination. According to this theory antigen molecules are specifically linked to one

another by polyvalent antibody molecules, and the formation of larger aggregates is as specific as the initial union of antigen to antibody, the whole process being continuous. The agglutination of spermatozoa would be explained as the linkage of antigen molecules on the tail of one to identical antigen molecules on another by specific polyvalent antibody molecules. In a system containing spermatozoa of different species and their respective agglutinating antibodies, aggregates each consisting entirely of spermatozoa of one species should be formed. Thus Topley, Wilson & Duncan (1935) showed that in mixtures of pneumococci and coliform bacilli and the corresponding antisera, monotypical aggregates were invariably obtained. The formation of mixed aggregates of spermatozoa suggests that their agglutination occurs in two stages, the specific union of antigen with antibody being followed by non-specific aggregation of the altered tails, whose surface has become sensitive to the flocculating action of electrolytes.

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#### DESCRIPTION OF PLATES 5 TO 7

The photomicrographs were taken with the Cooke 8 mm. phase contrast objective, except for figure 5 for which the Cooke 16 mm. phase contrast objective was used.

##### PLATE 5

FIGURE 1. Head agglutination in a suspension of rabbit epididymal spermatozoa, after 4 hr. in Baker's solution (magn.  $\times 530$ ).

FIGURE 2. Early agglutination of rabbit epididymal spermatozoa, after 2 min. in a 1 in 20 dilution of S. 1 (magn.  $\times 530$ ).

FIGURE 3. Agglutination of rabbit epididymal spermatozoa after 4 min. in a 1 in 20 dilution of S. 1 (magn.  $\times 530$ ).

##### PLATE 6

FIGURE 4. Agglutination of rabbit epididymal spermatozoa after 8 min. in a 1 in 20 dilution of S. 1 (magn.  $\times 530$ ).

FIGURE 5. Agglutination of rabbit epididymal spermatozoa after 8 min. in a 1 in 20 dilution of S. 1 (magn.  $\times 72$ ).

##### PLATE 7

FIGURE 6. Agglutination of rabbit, mouse, and guinea-pig spermatozoa after 2 min. in a 1 in 10 dilution of G. 2 (magn.  $\times 530$ ).

FIGURE 7. Agglutination of rabbit, mouse, and guinea-pig spermatozoa after 5 min. in a 1 in 10 dilution of G. 2 (magn.  $\times 530$ ).

# The theory of genetical recombination

## I. Long-chromosome arms

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This paper develops further the mathematical aspects of a theory of interference put forward recently in 'The sex chromosome in the house mouse' by Fisher, Lyon & Owen (1947). A mathematical model of a long chromosome is set up under simplifying assumptions, the principal one being that there exists a special *interference metric* specifying the positions of loci. It is assumed that the metrical lengths of the intercepts formed on a strand by the points of exchange of material have independent probability distributions.

Restricting attention to chromosome arms of great length, general analytical expressions are obtained for the quantities which are genetically observable. Under some additional assumptions special formulae are derived for map distance and recombination, in finite terms suitable for computation, and involving a disposable constant related to the intensity of the interference.

It is shown that with sufficiently intense interference the recombination fraction over a segment is an oscillatory function of the map length of the segment, and that recombinations in excess of 50 % are therefore in some cases to be expected.

### 1. INTRODUCTION

The present paper is intended both as a mathematical supplement to the recent note on 'The sex chromosome in the house mouse' (Fisher, Lyon & Owen 1947), and as the first of a series in which the ideas there put forward will be extended and applied, obtaining successive approximations to an adequate theory of interference and of the chromosome map.

One of the main aims of current genetical research is to determine in various organisms the topography of the genetical material, and to represent the mutable genes on each chromosome as occupying loci on a linear map. The order and spacing of the genes (though sometimes aided by cytological evidence and artificially produced rearrangements) has, mainly, to be inferred from the amounts of recombination exhibited by sets of genes when appropriately designed backcross experiments are carried out.

With closely spaced loci the recombination value  $y$  may itself be used as a conventional measure of distance (i.e. as a *metric*) in which to map the chromosome. But, in order to extend the map to wider intervals, it is necessary to introduce a more sophisticated convention. The *map distance*  $x$  between two loci is defined as the average number of points of exchange per strand which is formed between the loci in the course of a large number of meioses. This quantity is not directly observable, and is not equal to the recombination fraction  $y$ , which is the probability of occurrence of an odd number of exchange points. The inequality of  $x$  and  $y$  results from two causes, the occurrence of multiple crossing-over, and the operation of *interference*. With no interference, then, as pointed out by Haldane (1919),  $x$  and  $y$  are related by

$$2y = 1 - e^{-2x}.$$

Following other early work by Haldane (1919), Kosambi (1944) has proposed the formula

$$2y = \tanh 2x$$

as an approximate representation of the connexion between  $x$  and  $y$ , when there is interference of the intensity encountered in many organisms. If this formula holds, the recombination fraction over the sum of two contiguous intervals with fractions  $y_1, y_2$  is given by

$$y_{1+2} = \frac{y_1 + y_2}{1 + 4y_1y_2}.$$

Where data are available for three loci, this latter relation has been verified to hold to a fair degree of approximation, and so represents a considerable advance.

The position is, however, left unsatisfactory in three respects. In the first place the Kosambi formula sets an unpassable upper limit of 50 % to the recombination over any segment, and requires that  $y$  be a steadily increasing function of map length. But recombinations in excess of 50 % have been occasionally reported, and recently (Wright 1947) some good data of this kind have been obtained for the sex chromosome in the house mouse. Values of the order of 56 % were observed, and, more strikingly, two genes known to be widely separated (at a distance of the order of 30 cM) showed almost equal recombinations (in excess of 50 %) with a third locus not situated between them.

Secondly, Kosambi's formula predicates that the properties of the chromosome are uniform along its length; however, there is evidence of variation with distance from the centromere.

Thirdly, the Kosambi relation by itself does not provide a complete theory of gametic frequencies for linked loci, since with four genes or more the frequencies of the modes of gamete formation cannot be specified fully by its means. What is required, therefore, is a general formulation, in which may be specified, in terms of  $n$  parameters, the probability  $p_{r_1 r_2 \dots r_{n-1}}$  of there being exactly  $r_1, r_2, \dots, r_{n-1}$  points of exchange formed per strand in the intervals  $(1, 2), (2, 3), \dots, (n-1, n)$  between any number  $n$  of linked loci.

The present series of papers attempts this general formulation, on the basis of various simplifying assumptions which are necessary to make mathematical treatment possible. In this part attention is restricted to long-chromosome arms, and, as a first approximation, the assumption is made that such an arm is of infinite length. In part II this particular restriction will be removed.

It will be assumed that the centromere insulates from one another the two arms of a bivalent, so that chiasma formation upon one arm is independent of all events taking place upon the other. Each strand of an arm of a tetrad is idealized into a semi-infinite continuum of point loci, which may be regarded as mapped out into a semi-infinite straight line having its origin at the centromere  $O$ . A point on the strand may thus be defined in terms of its distance  $u$  from  $O$ , where  $u$  is measured in some metric yet to be specified.

This metric, in terms of which the interference properties of the chromosome will be specified, is not necessarily a linear function of the physical length of the chro-



matid, and must be distinguished from the cytological, mutational or genetical map distances.

Attention will be confined to events upon a single strand of the tetrad, since this is all that is relevant to purely genetical observations on diploids. Under certain further assumptions will be calculated the distribution and frequency of occurrence, on such a strand, of the points of exchange of material with non-sister strands. The distribution of points of exchange, once known, affords the material for development of analytical expressions for the genetical observables (such as map distance and recombination) in terms of the metrical parameters of loci.

At the outset it is assumed, for ease of exposition and conceptual simplicity, that the formation of chiasmata follows the serial process in space and time postulated by Mather (1936, 1937). In part II it will be shown that such an assumption is not logically essential to the probability calculus derived therefrom, and if desired may be dispensed with. The question of 'axiomatics' will be treated in a later part.

At present, following Mather's ideas, the point of exchange which on a particular strand is formed proximal to the centromere will be called the *first exchange point*. Let the probability that this first exchange point be formed in the elementary segment of metrical length  $du$ , situated at metrical distance  $u$  from the centromere, be

$$dp = f_1(u) du.$$

It is next postulated that, when the first exchange point has been established, the *second* point of exchange (proximal to the first) is formed, at some later time, in an interval  $dv$  at a distance  $v$  from the first, with a probability, conditional on the establishment of the former exchange point, which is given by

$$\begin{aligned} dp &= f_1(v) dv & (v > 0), \\ &= 0 & (v < 0). \end{aligned}$$

Similarly, it is assumed that the distribution of the  $r$ th exchange point conditional on the establishment of the  $(r-1)$ th is given by

$$\begin{aligned} dp &= f_1(w) dw & (w > 0), \\ &= 0 & (w < 0), \end{aligned}$$

$w$  denoting the distance between the  $(r-1)$ th and the  $r$ th chiasma.

It will be seen that the above postulates are equivalent to the two independent statements:

- (1) the distributions of intercept lengths between consecutive points of exchange are independent of one another;
- (2) interference properties of the centromere and of each of the established exchange points are identical on the metric chosen.

The various possible contributory mechanisms, namely, chiasma interference, chromatid interference, and bivalent competition, may be regarded as having their effects subsumed in the function  $f_1(u)$ , to which, later, plausible forms will be given.

For mathematical convenience it will be assumed in the sequel that  $f_1(u)$  is piecewise continuous.

The basic function  $f_1(u)$  may be regarded as having the following genesis. Suppose, at a given stage in the serial process of formation, that  $E$  is the point of exchange most

recently created and therefore is that one most distant from the centromere. Considering an elementary segment  $P$  of length  $du$  situated at a distance  $u$  beyond  $E$ , let it be supposed that the probability of an exchange point in  $du$ , conditional on there being no exchange point in the interval  $EP$ , is equal to

$$k(u) du.$$

It follows that the probability that no exchange point be formed in  $EP$  is

$$\prod_{v=0}^u \{1 - k(v) dv\} = \exp \left\{ - \int_0^u k(v) dv \right\}, \quad (1)$$

which we denote by  $p(u)$ ,

this formula resulting from the fact that the probability of no exchange in  $dv$ , conditional on there being no point of exchange between  $E$  and  $dv$ , is

$$1 - k(v) dv.$$

Thus the probability that there be a point of exchange formed in  $du$  and none in  $EP$  is the product

$$\begin{aligned} k(u) du p(u) &= k(u) \exp \left\{ - \int_0^u k(v) dv \right\} du \\ &= -du \frac{\partial}{\partial u} \exp \left\{ - \int_0^u k(v) dv \right\} \\ &= -du \frac{\partial}{\partial u} p(u) \\ &= d\{-p(u)\}. \end{aligned}$$

But this probability is merely the probability that the first exchange point following  $E$  should fall in  $du$ , and consequently the distribution of this point, conditional on  $E$ , is

$$dp = f_1(u) du = d\{-p(u)\}. \quad (2)$$

The condition, that the establishment of an exchange point completely inhibits other points in its immediate neighbourhood, is

$$f_1(0) = 0. \quad (3)$$

Also  $p(0) = 1$ , (4)

as is clear from the meaning of  $p(u)$  or from formula (1). It is further assumed that on the long-chromosome arm at least one exchange point is formed per strand. This requires that the probability of no points of exchange between the centromere and infinity be zero, that is

$$p(\infty) = 0.$$

It is assumed that the metric may be so chosen that, in the absence of interference, exchange points would appear with uniform prior probability so that

$$k(u) = k \quad \text{independent of } u.$$

In this case  $p(u) = e^{-ku}$ , which is the term of zero order in the Poisson series. Assuming that interference disappears at great distances, the limiting form of  $p(u)$  must be of this type. Hence

$$p(u) \sim e^{-ku} \quad \text{as } u \rightarrow \infty,$$

and

$$f_1(u) \sim k e^{-ku},$$

for some constant  $k$ .

We are at liberty to choose a conventional unit in which to measure the metric  $u$  in such a way that the mean distance between successive exchange points is  $\bar{u} = 1$ . For, if in any given scale

$$\bar{u} = \int_0^\infty u d\{-p(u)\} = 1/c,$$

the scale transformation

$$u^* = cu, \quad p^*(cu) = p(u),$$

gives

$$\bar{u}^* = \int_0^\infty cu d\{-p(u)\} = 1.$$

A metric of this type, for which  $\bar{u} = 1$ , will be said to be 'standardized' or in 'standard measure'.

## 2. FUNDAMENTAL FORMULAE

Denote by  $dp = f_r(u) du$  the distribution of the  $r$ th point of exchange,  $u$  being metrical distance from the centromere.

$$\text{Then} \quad f_2(u) = \int_0^u f_1(v) f_1(u-v) dv, \quad f_r(u) = \int_0^u f_{r-1}(v) f_1(u-v) dv. \quad (5)$$

Represent by  $F(\lambda, u)$  the generating function

$$F(\lambda, u) = \sum_{r=1}^{\infty} \lambda^{r-1} f_r(u).$$

The probability of a point of exchange being formed in  $du$  is

$$\sum_{r=1}^{\infty} f_r(u) du = F(1, u) du.$$

Denote by  $p_r(t)$  the probability of exactly  $r$  exchange points in the interval  $(0, t)$ , that is to say, in the segment with termini  $u = 0$  and  $u = t$ . Then

$$\left. \begin{aligned} p_r(t) &= \int_0^t f_r(u) p(t-u) du \quad (r \geq 1), \\ p_0(t) &= p(t) = \int_t^\infty f_1(u) du = 1 - \int_0^t f_1(u) du. \end{aligned} \right\} \quad (6)$$

Introducing the generating function  $P(\lambda, t) = \sum_{r=0}^{\infty} \lambda^r p_r(t)$  we have the identity

$$P(\lambda, t) = 1 + (\lambda - 1) \int_0^t F(\lambda, u) du, \quad (7)$$

which may be proved by writing

$$\frac{\partial}{\partial t} P(\lambda, t) = \sum_{r=0}^{\infty} \lambda^r \frac{\partial}{\partial t} p_r(t) = \frac{\partial}{\partial t} p(t) + \sum_{r=1}^{\infty} \lambda^r \frac{\partial}{\partial t} \int_0^t f_r(u) p(t-u) du.$$

Since 
$$\frac{\partial}{\partial t} \int_0^t f_r(u) p(t-u) du = f_r(t) p(t-t) + \int_0^t f_r(u) \frac{\partial}{\partial t} p(t-u) du$$

and 
$$p(0) = 1, \quad \frac{\partial}{\partial t} p(t-u) = -f_1(t-u),$$

we obtain 
$$\begin{aligned} \frac{\partial}{\partial t} P(\lambda, t) &= \sum_{r=1}^{\infty} \lambda^r f_r(t) - f_1(t) - \sum_{r=1}^{\infty} \lambda^r \int_0^t f_r(u) f_1(t-u) du \\ &= \sum_{r=1}^{\infty} \lambda^r f_r(t) - \sum_{r=0}^{\infty} \lambda^r f_{r+1}(t) \\ &= (\lambda - 1) F(\lambda, t), \end{aligned}$$

and the identity follows since  $P(\lambda, 0) = 1$ .

As corollary we have  $\sum_{r=1}^{\infty} p_r(t) = P(1, t) = 1$  for segments of all lengths showing that the system of probabilities is consistent.

By (5) we have

$$\begin{aligned} \lambda \int_0^t F(\lambda, t-u) f_1(u) du &= \lambda \int_0^t F(\lambda, u) f_1(t-u) du \\ &= \sum_{r=1}^{\infty} \lambda^r \int_0^t f_r(u) f_1(t-u) du = \sum_{r=1}^{\infty} \lambda^r f_{r+1}(t) \\ &= F(\lambda, t) - f_1(t), \end{aligned}$$

so that

$$\begin{aligned} F(\lambda, t) &= f_1(t) + \lambda \int_0^t F(\lambda, u) f_1(t-u) du \\ &= f_1(t) + \lambda \int_0^t F(\lambda, t-u) f_1(u) du, \end{aligned} \quad (8)$$

which are Volterra equations, defining  $f_1(t)$  and  $F(\lambda, t)$ , each uniquely in terms of the other. The functions  $\lambda F(\lambda, t-u)$  and  $-\lambda f_1(t-u)$  are reciprocal kernels. In particular, we have the inverse series

$$f_1(t) = \sum_{r=1}^{\infty} \lambda^{r-1} (-)^{r-1} F_r(\lambda, t),$$

where

$$\begin{aligned} F_1(\lambda, t) &= F(\lambda, t), \\ F_r(\lambda, t) &= \int_0^t F_{r-1}(\lambda, u) F_1(\lambda, t-u) du \quad (r \geq 2). \end{aligned}$$

We may note that if the expected density of exchange points  $F(1, t)$  is given, then  $f_1(t)$  is determined as  $\sum_{r=1}^{\infty} F_r(1, t)$ . It is easy to derive relations in terms of characteristic functions.

The transforms

$$M(\lambda, z) = \int_0^{\infty} e^{zt} F(\lambda, t) dt, \quad m(z) = \int_0^{\infty} e^{zt} f_1(t) dt$$

are certainly defined in the negative half-plane of  $z$ . From (8)

$$\begin{aligned}
 M(\lambda, z) &= m(z) + \lambda \int_0^\infty e^{zt} \int_0^t F(\lambda, t-u) f_1(u) du dt \\
 &= m(z) + \lambda \int_0^\infty du \int_u^\infty e^{zt} F(\lambda, t-u) f_1(u) du dt \quad \text{by Dirichlet's formula} \\
 &= m(z) + \lambda \int_0^\infty f_1(u) du \int_0^\infty e^{z(t+u)} F(\lambda, t) dt \\
 &= m(z) + \lambda \int_0^\infty e^{zu} f_1(u) du \int_0^\infty e^{zt} F(\lambda, t) dt \\
 &= m(z) + \lambda m(z) M(\lambda, z),
 \end{aligned}$$

so that

$$M(\lambda, z) = m(z) / \{1 - \lambda m(z)\}. \quad (9)$$

Substituting two values  $\lambda_1, \lambda_2$  in (9) and eliminating  $m(z)$  we obtain

$$\frac{1}{M(\lambda_2, z)} - \frac{1}{M(\lambda_1, z)} = \lambda_1 - \lambda_2$$

or

$$M(\lambda_1, z) - M(\lambda_2, z) = (\lambda_1 - \lambda_2) M(\lambda_1, z) M(\lambda_2, z),$$

which inverts to give the integral relation

$$(\lambda_1 - \lambda_2) \int_0^t F(\lambda_1, t-u) F(\lambda_2, u) du = F(\lambda_1, t) - F(\lambda_2, t), \quad (10)$$

with the corollary

$$\int_0^t F(\lambda, t-u) F(\lambda, u) du = \frac{\partial}{\partial \lambda} F(\lambda, t). \quad (11)$$

For the general segment  $(t_1, t_2)$  whose termini are the loci  $u = t_1, u = t_2$  ( $> t_1$ ) we define  $p_q(t_1, t_2)$  as the probability of  $q$  exchange points and construct the generating function

$$P(\lambda, t_1, t_2) = \sum_{q=0}^{\infty} \lambda^q p_q(t_1, t_2).$$

Denote by  $f_q(u | r, t_1)$  the probability density at  $u$  of the  $q$ th point of exchange in  $(t_1, t_2)$  conjoint with there being  $r$  such points in  $(0, t_1)$  so that

$$\left. \begin{aligned}
 f_q(u | r, t_1) &= f_1(u) & (r=0, q=1) \\
 &= \int_0^{t_1} f_r(v) f_1(u-v) dv & (r \geq 1, q=1) \\
 &= \int_{t_1}^u f_{q-1}(v | r, t_1) f_1(u-v) dv & (r \geq 0, q \geq 2).
 \end{aligned} \right\} \quad (12)$$

Writing

$$F(\lambda, u, t) = \sum_{q=1}^{\infty} \sum_{r=0}^{\infty} \lambda^{q-1} f_q(u | r, t_1),$$

then by (12)

$$F(\lambda, u, t_1) = f_1(u) + \int_0^{t_1} F(1, v) f_1(u-v) dv + \lambda \int_{t_1}^u F(\lambda, v, t_1) f_1(u-v) dv \quad (13)$$

$$= z(u, t_1) + \lambda \int_{t_1}^u F(\lambda, v, t_1) f_1(u-v) dv, \quad (14)$$

with

$$z(u, t) = f_1(u) + \int_0^t F(1, v) f_1(u - v) dv.$$

It may be shown that

$$F(\lambda, u, t) = z(u, t) + \lambda \int_t^u F(\lambda, u - v) z(v, t) dv \quad (15)$$

$$= F(\lambda, u) + (1 - \lambda) \int_0^t F(1, v) F(\lambda, u - v) dv. \quad (16)$$

For, since  $\lambda F(\lambda, u - v)$  and  $-\lambda f_1(u - v)$  are reciprocal kernels, it follows from the Volterra theory (Bôcher 1913) that the unique continuous solution of (14) is

$$F(\lambda, u, t_1) = z(u, t_1) + \lambda \int_{t_1}^u F(\lambda, u - v) z(v, t_1) dv.$$

And, differentiating this equation,

$$\frac{\partial F}{\partial t_1} = \frac{\partial}{\partial t_1} z(u, t_1) - \lambda F(\lambda, u - t_1) z(t_1, t_1) + \lambda \int_{t_1}^u F(\lambda, u - v) \frac{\partial}{\partial t_1} z(v, t_1) dv.$$

But

$$\frac{\partial}{\partial t} z(u, t) = F(1, t) f_1(u - t)$$

and

$$z(t, t) = f_1(t) + \int_0^t F(1, v) f_1(t - v) dv = F(1, t).$$

Therefore

$$\begin{aligned} \frac{\partial}{\partial t} F(\lambda, u, t) &= F(1, t) \left\{ -\lambda F(\lambda, u - t) + f_1(u - t) + \lambda \int_t^u F(\lambda, u - v) f_1(v - t) dv \right\} \\ &= F(1, t) F(\lambda, u - t) (1 - \lambda), \end{aligned}$$

since

$$f_1(u - t) + \lambda \int_t^u F(\lambda, u - v) f_1(v - t) dv = F(\lambda, u - t).$$

Also

$$\begin{aligned} F(\lambda, u, 0) &= f_1(u) + \lambda \int_0^u F(\lambda, u - v) f_1(v) dv \quad \text{by (15)} \\ &= F(\lambda, u), \end{aligned}$$

so that (16) follows on integration.

$P(\lambda, t_1, t_2)$  may now be computed in terms of the auxiliary function  $F(\lambda, u, t_1)$  which has itself been expressed in terms of the basic function  $F(\lambda, u)$ .

We have

$$p_0(t_1, t_2) = p(t_2) + \int_0^{t_1} \sum_{r=1}^{\infty} f_r(v) p(t_2 - v) dv, \quad p_q(t_1, t_2) = \sum_{r=0}^{\infty} \int_{t_1}^{t_2} f_q(v | r, t_1) p(t_2 - v) dv,$$

so that

$$P(\lambda, t_1, t_2) = p(t_2) + \int_0^{t_1} F(1, v) p(t_2 - v) dv + \lambda \int_{t_1}^{t_2} F(\lambda, v, t_1) p(t_2 - v) dv.$$

Therefore

$$\frac{\partial P}{\partial t_2} = -f_1(t_2) - \int_0^{t_1} F(1, v) f_1(t_2 - v) dv + \lambda F(\lambda, t_2, t_1) - \lambda \int_{t_1}^{t_2} F(\lambda, v, t_1) f_1(t_2 - v) dv,$$

since

$$\frac{\partial}{\partial t} p(t) = -f_1(t) \quad \text{and} \quad p(0) = 1.$$

Hence 
$$\frac{\partial P}{\partial t_2} = -F(\lambda, t_2, t_1) + \lambda F(\lambda, t_2, t_1) \quad \text{by (13)}$$
$$= (\lambda - 1) F(\lambda, t_2, t_1).$$

Integrating, we obtain

$$P(\lambda, t_1, t_2) = 1 + (\lambda - 1) \int_{t_1}^{t_2} F(\lambda, u, t_1) du, \quad (17)$$

which gives, in particular

$$\sum_{q=0}^{\infty} p_q(t_1, t_2) = P(1, t_1, t_2) = 1,$$

showing that the sum of the probabilities is unity.

We may note also the formula

$$P(\lambda, t_1, t_2) = 1 + (\lambda - 1) \left\{ \int_0^{t_2} F(\lambda, u) du - \int_0^{t_1} x'(u) P(\lambda, t_2 - u) du \right\},$$

which obtains on account of the identity

$$\int_0^t F(\lambda, u) du = \int_0^t F(1, u) P(\lambda, t - u) du,$$

which is the integral of

$$F(\lambda, t) - F(1, t) = (\lambda - 1) \int_0^t F(1, u) F(\lambda, t - u) du.$$

### 3. GENETICAL MAP DISTANCE AND RECOMBINATION FRACTION

The quantities of interest are those which are observable genetically, and these include the map distance between and the recombination fraction of two loci. The recombination fraction is directly in evidence in linkage crosses and the map distance is an observable in a less direct sense, being approximated to by the sum of the recombination fractions, in successive pairs, of closely spaced genes forming a chain between the two loci in question.

In terms of the exchange point probabilities, as defined in the preceding section,

$$x(t) = \sum_{r=0}^{\infty} r p_r(t), \quad (18)$$

$$y(t) = \sum_{r=0}^{\infty} p_{2r+1}(t), \quad (19)$$

denoting the map distance and recombination fraction for the interval  $(0, t)$  proximal to the centromere, which are defined respectively as the expected number of points of exchange in the interval and as the probability of an odd number of such points occurring.

We have the relations

$$x(t) = \sum_{r=0}^{\infty} r p_r(t) = \bar{r} = \left. \frac{\partial}{\partial \lambda} P(\lambda, t) \right|_{\lambda=1}, \quad (20)$$

$$y(t) = \sum_{r=0}^{\infty} p_{2r+1}(t) = \frac{1}{2} \left\{ 1 - \sum_{r=0}^{\infty} (-)^r p_r(t) \right\} = \frac{1}{2} \{ 1 - P(-1, t) \}. \quad (21)$$

In virtue of  $P(\lambda, t) = 1 + (\lambda - 1) \int_0^t F(\lambda, u) du$  it follows that

$$\left. \begin{aligned} x(t) &= \int_0^t F(1, u) du, & x'(t) &= F(1, t), \\ y(t) &= \int_0^t F(-1, u) du, & y'(t) &= F(-1, t), \end{aligned} \right\} \quad (22)$$

and therefore

$$x'(t) = f_1(t) + \int_0^t x'(t-u) f_1(u) du, \quad y'(t) = f_1(t) - \int_0^t y'(t-u) f_1(u) du, \quad (23)$$

together with  $2 \int_0^t x'(t-u) y'(u) du = x'(t) - y'(t) \quad \text{by (10),} \quad (24)$

and 
$$\left. \begin{aligned} x(t) &= 1 - p(t) + \int_0^t x(t-u) f_1(u) du, \\ y(t) &= 1 - p(t) - \int_0^t y(t-u) f_1(u) du, \\ 2 \int_0^t x(t-u) y'(u) du &= 2 \int_0^t x'(t-u) y(u) du \\ &= x(t) - y(t), \end{aligned} \right\} \quad (25)$$

which follow from (23) and (24) by integration.

It is easily verified that the map distance  $x(t)$  is proportional to the metric  $t$ , only in the case of non-interfering and, therefore, totally random occurrence of points of exchange. For, by (23), the assumption  $x(t) = kt$  requires

$$k = f_1(t) + k \int_0^t f_1(u) du,$$

which has the unique solution  $f_1(t) = k e^{-kt}$ . Substituting this form in the latter of equations (23) and solving, we get, for  $y$ , the Haldane formula for zero interference,

$$y(t) = \frac{1}{2}(1 - e^{-2kt}) = \frac{1}{2}(1 - e^{-2t}).$$

It may be verified that, for all sufficiently smooth forms of the distribution function  $f_1(u)$ , the recombination value with the centromere as the locus at  $t$  recedes to infinity tends to the non-linkage value of  $\frac{1}{2}$ . Rearranging the second of equations (25) we obtain

$$\lim_{t \rightarrow \infty} \left\{ y(t) + \int_0^t f_1(t-u) y(u) du \right\} = \lim_{t \rightarrow \infty} \{1 - p(t)\} = 1. \quad (26)$$

The conditions of the Mercerian theorem of Paley & Wiener (1934) are satisfied, because  $\int_0^\infty |f_1(u)| du$  exists, being equal to  $\int_0^\infty f_1(u) du = 1$  and

$$m(z) = \int_0^\infty e^{zu} f_1(u) du \neq -1 \quad \text{if} \quad \text{Re } z \leq 0.$$



For, if  $z = -\xi + i\eta$  and  $\xi > 0$ , then

$$|m(z)| \leq \int_0^\infty e^{-\xi u} f_1(u) du < 1,$$

and, if  $z = i\eta$ ,

$$|\mathbf{R}m(z)| = \left| \int_0^\infty \cos \eta u f_1(u) du \right| \leq \int_0^\infty f_1(u) |\cos \eta u| du < \int_0^\infty f_1(u) du = 1,$$

unless  $\eta = 0$ , but then  $m(z) = 1$ .

The conditions of the theorem being satisfied, (26) then implies

$$\lim_{t \rightarrow \infty} y(t) = \left\{ 1 + \int_0^\infty f_1(u) du \right\}^{-1} = \frac{1}{2}.$$

For the map distance we may note that its derivative  $x'(t)$ , being equal to

$$F(1, t) = \sum_{r=0}^\infty f_r(t),$$

is essentially positive, so that  $x(t)$  steadily increases with  $t$  and consequently tends either to a finite limit  $X$  or to infinity. On the former hypothesis we would have

$$\begin{aligned} X &= \lim \left\{ 1 - p(t) + \int_0^t x(t-u) f_1(u) du \right\} \\ &= 1 + \lim \int_0^t x(t-u) f_1(u) du \\ &= 1 + X \int_0^\infty f_1(u) du, \end{aligned}$$

by the integral analogue of Tannery's theorem, so that we arrive at the contradiction  $X = 1 + X$ .

Hence  $x(t)$  tends to infinity with  $t$ .

It is natural to suppose that for long segments of metric length  $t$ , the mean intercept length  $\bar{u} = \mu_1$  is given approximately by  $t/x(t)$ , and therefore that  $x(t) \sim t/\mu_1$  as  $t \rightarrow \infty$ .

However, a more precise and general result may be established, namely, if

$$f_1(u) \sim g_1(u) \quad \text{as } u \rightarrow \infty, \quad (27)$$

where  $g_1(u) = \frac{a^{1+b} u^b e^{-au}}{\Gamma(1+b)}$ , with  $a > 0$ ,  $b \geq 0$ , then

$$x(t) = \frac{1}{\mu_1} t - \left( 1 - \frac{\mu_2}{2\mu_1^2} \right) + o(1), \quad (28)$$

where  $\mu_1, \mu_2$  are the first two moments of  $f_1(u)$ .

We note that  $x'(t)$  is bounded, for, if we assume

$$f_r(u) = g_r(u) + h_r(u),$$

then

$$f_{r+1}(t) = \int_0^t f_r(u) f_1(t-u) du$$

is given by

$$\begin{aligned}
 & \int_{\frac{1}{2}t}^t f_r(u) f_1(t-u) du + \int_{\frac{1}{2}t}^t f_1(u) f_r(t-u) du \\
 &= \int_{\frac{1}{2}t}^t g_r(u) g_1(t-u) du + \int_{\frac{1}{2}t}^t g_1(u) g_r(t-u) du + H_r(t) \quad \text{say,} \\
 &= \int_0^t g_r(u) g_1(t-u) du + H_r(t) \\
 &= g_{r+1}(t) + h_{r+1}(t),
 \end{aligned}$$

and we can prove, on carrying out the induction in detail, that  $\sum_{r=1}^{\infty} h_r(t) = O(1)$  and, therefore,  $x'(t) = \sum_{r=1}^{\infty} g_r(t) + O(1)$ , which is bounded.

Consequently, the transform  $\int_0^{\infty} e^{zt} x(t) dt$  exists in the region  $\operatorname{Re} z < 0$ , where

$$\begin{aligned}
 \int_0^{\infty} e^{zt} x(t) dt &= -\frac{1}{2} \int_0^{\infty} e^{zt} x'(t) dt \quad \text{by partial integration} \\
 &= -\frac{1}{2} M(1, z) = -\frac{1}{2} \frac{m(z)}{1-m(z)} \quad \text{by (19).}
 \end{aligned}$$

$m(z) = \int_0^{\infty} e^{zt} f_1(t) dt$  has an expansion  $1 + \mu_1 z + \mu_2 z^2 + \dots$  in the neighbourhood of the origin, and  $\mu_1, \mu_2$  are not zero. It follows that the function defined from  $\int_0^{\infty} e^{zt} x(t) dt$  has a Laurent expansion

$$-\frac{1}{\mu_1} \frac{1}{z^2} + \left(1 - \frac{\mu_2}{2\mu_1^2}\right) \frac{1}{z} + \alpha_1 + \alpha_2 z^2 + \dots$$

On account of (27)  $m(z)$  is regular in  $\operatorname{Re} z \leq a$  and in this region  $-M(1, z)/z$  has no singularities other than poles at the zeroes of  $z\{m(z) - 1\}$ . The origin is such a zero, but for all other  $z$  in  $\operatorname{Re} z < a$ , we have  $|m(z)| < \int_0^{\infty} f_1(u) du = 1$ . Consequently the function

$$L_1(z) = -\frac{1}{2} M(1, z) + \frac{1}{\mu_1} \frac{1}{z^2} - \left(1 - \frac{\mu_2}{2\mu_1^2}\right) \frac{1}{z}$$

is regular in the whole of some region  $\operatorname{Re} z \leq a'$  where  $a' < a$ . Since, also,

$$L_1(z) = \int_0^{\infty} e^{zt} \left\{ x(t) - \frac{1}{\mu_1} t + \left(1 - \frac{\mu_2}{2\mu_1^2}\right) \right\} dt,$$

the Laplace inversion formula gives

$$x(t) - \frac{1}{\mu_1} t + \left(1 - \frac{\mu_2}{2\mu_1^2}\right) = \frac{1}{2\pi i} \int_{c-i\infty}^{c+i\infty} L_1(z) e^{-zt} dz, \quad (29)$$

where  $c < 0$ . It is easy to see that if  $\operatorname{Re} z \leq 0$  then  $|L_1(z)| = o(1)$ , as the imaginary part of  $z$  tends to infinity. For, if  $z = -\xi + i\eta$ ,

$$m(z) = \int_0^{\infty} e^{-\xi t} f_1(t) e^{i\eta t} dt = o(1)$$

as  $\eta \rightarrow \infty$ , by the Riemann-Lebesgue theorem, and therefore  $L(z) = o(1)$  also. Hence the contour of integration in (29) may be shifted to the imaginary axis, giving

$$\begin{aligned} x(t) - \frac{1}{\mu_1} t + \left(1 - \frac{\mu_2}{2\mu_1^2}\right) &= \frac{1}{2\pi i} \int_{-i\infty}^{i\infty} L_1(z) e^{-zt} dz \\ &= \frac{1}{2\pi} \int_{-\infty}^{\infty} L(i\eta) e^{-i\eta t} d\eta \\ &= o(1) \quad \text{as } t \rightarrow \infty, \end{aligned}$$

again in virtue of the Riemann-Lebesgue theorem.

If the metric is in standard measure, then  $\mu_1 = 1$  and

$$\begin{aligned} x(t) &= t - (1 - \tfrac{1}{2}\mu_2) + o(1) \\ &= t - \tfrac{1}{2}(1 - \text{var} f_1) + o(1), \end{aligned} \tag{30}$$

where  $\text{var} f_1$  denotes the variance of  $f_1(u)$ .

A similar argument may, of course, be employed to establish the previous result that

$$y(t) = \tfrac{1}{2} + o(1) \quad \text{and also that} \quad P(\lambda, t) = o(1) \quad \text{for all } |\lambda| < 1.$$

For the general segment we have, with an obvious notation,

$$\begin{aligned} x(t_1, t_2) &= \sum_{q=0}^{\infty} q p_q(t_1, t_2) = \frac{\partial}{\partial \lambda} P(\lambda, t_1, t_2) \Big|_{\lambda=1} \\ &= \int_{t_1}^{t_2} F(1, u, t_1) du, \quad \text{by (17)}. \end{aligned} \tag{31}$$

But  $F(1, u, t_1) = F(1, u)$  by (16), and therefore

$$x(t_1, t_2) = \int_{t_1}^{t_2} F(1, u) du = x(t_2) - x(t_1) \tag{32}$$

as is to be expected, map length being simply the integral of density of exchange points. Also, for recombination,

$$\begin{aligned} y(t_1, t_2) &= \sum_{q=0}^{\infty} p_{2q+1}(t_1, t_2) = \tfrac{1}{2}\{1 - P(-1, t_1, t_2)\} \\ &= \int_{t_1}^{t_2} F(-1, u, t_1) du, \end{aligned} \tag{33}$$

which is not the simple difference of  $y(t_2)$  and  $y(t_1)$ , the effect of interference being shown through the relations

$$\begin{aligned} F(-1, u, t_1) &= F(-1, u) + 2 \int_0^{t_1} F(1, v) F(-1, u-v) dv \\ &= y'(u) + 2 \int_0^{t_1} x'(v) y'(u-v) dv, \end{aligned}$$

where  $y'(w)$  is now employed in a slightly wider sense than formerly, as representing  $F(-1, w)$ . Thus

$$y(t_1, t_2) = y(t_2) - y(t_1) + 2 \int_{t_1}^{t_2} du \int_0^{t_1} x'(v) y'(u-v) dv \quad (34)$$

$$\begin{aligned} &= y(t_2) - x(t_1) + 2 \int_0^{t_1} x'(v) y(t_2-v) dv \\ &= y(t_2) - 2 \int_0^{t_1} x'(v) \left\{ \frac{1}{2} - y(t_2-v) \right\} dv. \end{aligned} \quad (35)$$

To establish these last relations we note that

$$\begin{aligned} 2 \int_{t_1}^{t_2} du \int_0^{t_1} x'(v) y'(u-v) dv &= 2 \int_0^{t_1} x'(v) \int_{t_1}^{t_2} y'(u-v) du dv \\ &= 2 \int_0^{t_1} x'(v) \{y(t_2-v) - y(t_1-v)\} dv, \quad \text{and apply (24).} \end{aligned}$$

Equation (35) exemplifies the fact that a proper formulation of a theory of interference excludes an addition theorem for the recombination fractions of segments such as exists in the empirical Kosambi theory.

For two segments terminating at the centromere and on *opposite* sides of it, we have

$$x_{12} = x_1 + x_2, \quad y_{12} = y_1 + y_2 - 2y_1y_2,$$

since the generating function of probabilities is the product of the functions appropriate to the constituent segments, the arms having been assumed independent.

Equation (35) shows that as  $t_2$  recedes to infinity  $y(t_1, t_2)$  tends to  $\frac{1}{2}$ , which is the value for independent segregation. For

$$\begin{aligned} \lim_{t_2 \rightarrow \infty} y(t_1, t_2) &= \lim_{t_2 \rightarrow \infty} y(t_2) - 2 \int_0^{t_1} x'(v) \left[ \lim_{t_2 \rightarrow \infty} \left\{ \frac{1}{2} - y(t_2-v) \right\} \right] dv \\ &= \frac{1}{2} - 2 \int_0^{t_1} x'(v) 0 dv \\ &= \frac{1}{2}. \end{aligned}$$

#### 4. SPECIAL FORMULAE FOR MAP LENGTH AND RECOMBINATION

To obtain formulae suitable for computation and comparison with observation it is necessary to particularize the function  $f_1(u)$ . Any choice of a form for this function can clearly only receive posterior justification based on a correspondence of theoretical prediction with observation. In their discussion of the sex chromosome in the mouse Fisher *et al.* (1947) suggested as a plausible first approximation

$$f_1(u) = \frac{\partial}{\partial u} \{ -\operatorname{sech} \frac{1}{2}\pi u \} = \frac{1}{2}\pi \tanh \frac{1}{2}\pi u \operatorname{sech} \frac{1}{2}\pi u,$$

satisfying

$$f_1(0) = 0, \quad f_1(u) \sim \frac{1}{2}\pi e^{-\frac{1}{2}\pi u} \quad \text{as } u \rightarrow \infty,$$

$$\int_0^\infty f_1(u) du = 1, \quad \bar{u} = \int_0^\infty u f_1(u) du = 1,$$

and with variance  $\text{var} f_1 = 0.4849$ . The corresponding functions  $p(u)$  and  $k(u)$  are

$$p(u) = \text{sech } \frac{1}{2}\pi u, \quad k(u) = \tanh \frac{1}{2}\pi u,$$

giving to the theory a certain uniformity. Apart from the question of posterior justification this form was suggested by analogy with the Kosambi functional relation

$$2y = \tanh 2x,$$

which has so far been the most useful representation of the effects of interference.

Clearly, there can be no universally valid form of  $f_1(u)$  applying to all chromosomes of all species. To determine an approximate form for a particular chromosome in a given species would require a vast body and amount of data from linkage experiments, such as probably hardly exist even in the case of the *Drosophila* linkage groups. While such fitting will be desirable, it will not be attempted at the present stage. Instead, the function  $\partial(-\text{sech } \frac{1}{2}\pi u)/\partial u$ , which appears in the case of the sex chromosome of *Mus musculus* to have been an apt choice, will be replaced by a close mimic of very simple mathematical properties, which is therefore suitable, at the least, for making a qualitative survey of the effects of interference. In part II, where coincidence is considered, it will be shown that the Kosambi formula represents an approximation to the results which proceed from the present assumptions.

If 
$$f_1(u) = 4u e^{-2u},$$

then 
$$m(z) = 1 + z + \frac{3}{4}z^2 + \dots = 1/(1 - \frac{1}{2}z)^2,$$

so that  $\bar{u} = 1$ ,  $\text{var} f_1 = \frac{1}{2}$  which is very close to the variance of

$$\frac{\partial}{\partial u} \{-\text{sech } \frac{1}{2}\pi u\}.$$

This choice of function has a defect, in that the order at infinity is slightly too great in respect of the factor  $u$ , which is equivalent to the implicit assumption of a residual interference operating at great distances. In the treatment of the finite chromosome arm to be developed later this is not even a theoretical hindrance, since the form of  $f_1(u)$  only needs to be defined over a finite range. Similarly, the order of magnitude at infinity fails, in the present stage of theory, to constitute a real ground of criticism. The purpose of the mathematical idealization which extends the strand to infinity is not, of course, to predict the distribution of points of exchange upon the remote imaginary portion, but rather to obtain an approximation to the form of this distribution for the proximal parts of the strand (a proximal part of the ideal infinite strand being identified with some real finite strand). The mathematical device of permitting the infinite extension is equivalent merely to assuming that the end of the arm exerts no repressive effect on the formation of exchange points. To this degree of approximation any observables calculated for a segment ending at  $u = t$ , are taken as being the values appropriate to a chromosome of this (or any greater) finite length.

For convenience in calculation an unstandardized metric will be employed in which  $f_1(u) = u e^{-u}$ . The final formulae will be obtained by replacing any metrical length  $t$  by  $2t$ . We obtain, by direct calculation,

$$f_1(u) = u e^{-u}, \quad f_2(u) = \frac{u^3}{3!} e^{-u}, \quad f_r(u) = \frac{u^{2r-1}}{(2r-1)!} e^{-u}, \quad (36)$$

so that the exchange-point density is

$$F(1, u) = e^{-u} \sum_1^{\infty} \frac{u^{2r-1}}{(2r-1)!} = e^{-u} \sinh u, \quad (37)$$

and 
$$F(\lambda, u) = e^{-u} \sum_1^{\infty} \frac{\lambda^{r-1} u^{2r-1}}{(2r-1)!} = e^{-u} \frac{\sinh u \sqrt{\lambda}}{\sqrt{\lambda}}. \quad (38)$$

For the probabilities of 0, 1, 2, ... points of exchange in  $(0, t)$  we have

$$p(t) = (1+t)e^{-t}, \quad p_1(t) = \left(\frac{t^2}{2!} + \frac{t^3}{3!}\right)e^{-t}, \quad p_2(t) = \left(\frac{t^4}{4!} + \frac{t^5}{5!}\right)e^{-t}, \dots,$$

which are the terms of a compound Poisson series (Feller 1943) (as is not unexpected since interference may be regarded as negative contagion).

The probability of at least one point of exchange on a chromosome arm of length  $T$  is

$$1 - p(T) = 1 - (1+T)e^{-T} = 1 - e^{-T} - Te^{-T}.$$

Integrating  $F(1, u) = e^{-u} \sinh u$  and  $F(-1, u) = e^{-u} \sin u$

from 0 to  $t$  we get 
$$x(t) = \frac{1}{2}t - \frac{1}{4} + \frac{1}{4}e^{-2t}, \quad (39)$$

$$y(t) = \frac{1}{2} - \frac{1}{2}e^{-t}(\cos t + \sin t). \quad (40)$$

Also, for the general segment  $(t_1, t_2)$ , we have, with the notation of § 2,

$$z(u, t_1) = (u - t_1) \cosh t_1 + \sinh t_1,$$

$$F(\lambda, u, t_1) = e^{-u} \left\{ \sinh t_1 \cosh \sqrt{\lambda} (u - t_1) + \cosh t_1 \frac{\sinh \sqrt{\lambda} (u - t_1)}{\sqrt{\lambda}} \right\},$$

so that, on integrating  $F(1, u, t_1)$  and  $F(-1, u, t_1)$ , we obtain

$$x(t_1, t_2) = x(t_2) - x(t_1) = \frac{1}{2}(t_2 - t_1) + \frac{1}{4}(e^{-2t_2} - e^{-2t_1}),$$

$$\begin{aligned} y(t_1, t_2) &= \frac{1}{2} - \frac{1}{2}e^{-t_2} \{e^{t_1} \cos(t_2 - t_1) + e^{-t_1} \sin(t_2 - t_1)\} \\ &= \frac{1}{2} - \frac{1}{2} \{e^{-(t_2 - t_1)} \cos(t_2 - t_1) + e^{-(t_2 + t_1)} \sin(t_2 - t_1)\}. \end{aligned}$$

Thus, in the standardized metric, we have the formulae

$$f_r(u) = 2 \frac{(2u)^{2r-1} e^{-u}}{(2r-1)!} \quad \text{Density of } r\text{th exchange point}, \quad (41)$$

$$F(1, u) = 1 - e^{-4u} \quad \text{Density of all exchange points (figure 2, } I = \frac{1}{2}), \quad (42)$$

$$x(t) = t - \frac{1}{4} + \frac{1}{4}e^{-4t} \quad \text{Map distance from centromere (figure 3, } I = \frac{1}{2}), \quad (43)$$

$$y(t) = \frac{1}{2} - \frac{1}{2}e^{-2t}(\cos 2t + \sin 2t) \quad \text{Recombination value with centromere (figure 4, } I = \frac{1}{2}), \quad (44)$$

$$x(t_1, t_2) = t_2 - t_1 + \frac{1}{4}(e^{-4t_2} - e^{-4t_1}) \quad (45)$$

and

$$y(t_1, t_2) = \frac{1}{2} - \frac{1}{2}e^{-2(t_2 - t_1)} \cos 2(t_2 - t_1) - \frac{1}{2}e^{-2(t_2 + t_1)} \sin 2(t_2 - t_1), \quad (46)$$

for the map distance and recombination fraction of two general loci.

It will be noted that the defect  $t-x(t)$  for long proximal segments has the value 0.25 or 25 cM in agreement with the magnitude  $\frac{1}{2}(1-\text{var}f_1)$  calculated in § 3, and that  $y(t_1, t_2)$  tends to 0.5 in all segments when the distal locus recedes to infinity.

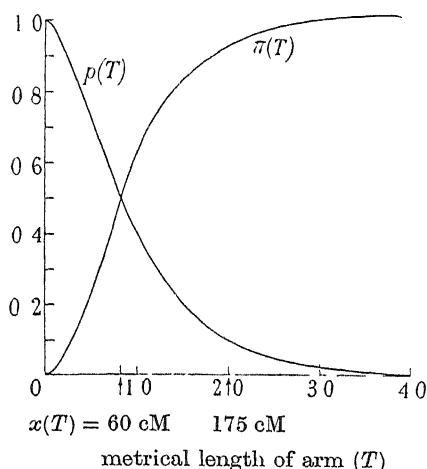


FIGURE 1. Probabilities of no points of exchange and of at least one point on arm of metrical length  $T$ .

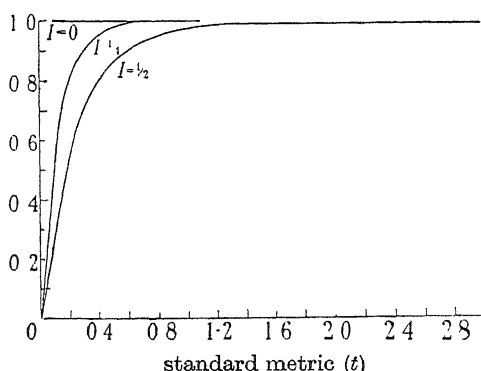


FIGURE 2. Expected density of points of exchange for various degrees of interference.

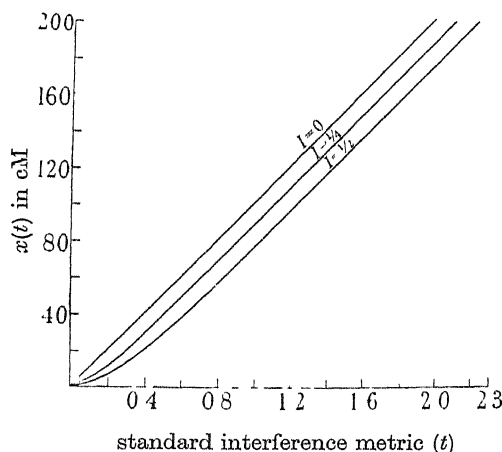


FIGURE 3. Map distance from centromere as function of metric for various degrees of interference.

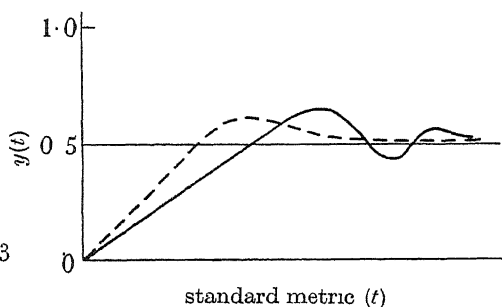


FIGURE 5. Sketch of possible modes of behaviour of the recombination fraction.

(Figure 4 appears on p. 89)

Neither of the genetical observables is a function of interval length only but depends on both the metrical distance between the loci, and on the location of the segment with respect to the centromere.

The existence of interference of the character considered here implies, on long segments, recombination values in excess of 50 %. The recombination value with the centromere  $y(t)$  takes, as we travel out from the centromere, alternately maximum

and minimum values, and oscillates about the ultimate 50 % level. The course of this function which is exhibited in figure 4, by the curve  $I = \frac{1}{2}$ , is a damped wave. Table 1 gives the positions and magnitudes of the nearer maxima and minima.

TABLE 1

$t$	$x(t)$ in cM	maxima of $y(t)$	minima of $y(t)$
$\frac{1}{2}\pi$	158.16	0.52161	—
$\pi$	289.21	—	0.49907
$\frac{3}{2}\pi$	446.24	0.50004	—
$2\pi$	603.32	—	$0.5 - 1.73 \times 10^{-6}$

Since chromosome lengths above 200 cM are rare, we do not attach much reality to the latter entries, but the first maximum, nevertheless, may be observable, though its position and magnitude are modified by the effect of the chromosome end, of which account is taken in part II.

If  $t_1$  is kept fixed, the positions of the distal locus for which the recombination value achieves maximum and minimum values are given by

$$t_2 = t_1 - \frac{1}{2} \tan^{-1}(\tanh 2t_1) + \frac{1}{2}n\pi, \quad (47)$$

and these values are

$$y_m = \frac{1}{2} \{1 + (-)^{n+1} e^{\{-n\pi - 2t_1 + \tan^{-1}(\tanh 2t_1)\}} \sqrt{(\cosh 4t_1)}\}, \quad (48)$$

which for large values of  $t_1$  tend to

$$y_m = \frac{1}{2} \left\{ 1 + (-)^{n+1} e^{-(n-\frac{1}{2})\pi} \frac{1}{\sqrt{2}} \right\},$$

the odd integral values of  $n$  defining the maxima and the even values the minima, all the maxima exceeding 0.5. When  $t_1 = 0$  the extreme values become merely

$$y_m = \frac{1}{2} \{1 + (-)^{n+1} e^{-n\pi}\}.$$

A graph of  $y(t) = y(0, t)$  as function of  $t$  will be found in figure 4, being the curve  $I = \frac{1}{2}$ .  $x(t)$ , the map distance from the centromere, is illustrated by figure 3.

Table 2 gives as percentages the values of  $y_{12}$  as a function of segment length  $(t_2 - t_1)$  for various positions of the first locus, namely, the centromere and at  $t_1 = 0.4$ ,  $t_1 = 0.8$  and  $t_1 = 1.2$ , which are respectively at map distances 0, 20.05, 56.02 and 95.21 cM from the centromere. The values, computed rather conveniently from Hayashi's tables (1930), have a possible error of  $\pm 1$  in the last figure.

The table shows that  $y_m(t_1)$ , the maximum of  $y_{12}$  for given  $t_1$ , increases as  $t_1$  is moved out. Also, the variation in  $y_{12}$  for changing  $x_{12}$  is extremely slight for  $x_{12}$  in excess of 60–70 cM, so that determination of position from loose linkage observations is very inaccurate.

In standardized metric the probability of at least one point of exchange on a chromosome arm of length  $T$  is

$$\pi(T) = 1 - p(T) = 1 - (1 + 2T) e^{-2T},$$

while that of no such point is  $(1 + 2T) e^{-2T}$ .



These probabilities are shown as functions of  $T$  in figure 1.  $\pi(T)$  is less than 0.5 for  $T < 0.85$  and therefore for arms shorter than 60 cM, but exceeds 0.9 on arms longer than 174 cM, so that within the present theory the probability of no exchange point is very appreciable on arms of short or moderate length.

TABLE 2. PERCENTAGE RECOMBINATION FRACTION OVER  $(t_1, t_2)$ 

interval ( $t_2 - t_1$ )	$y_{12}$ ( $t_1 = 0$ )	$y_{12}$ ( $t_1 = 0.4$ )	$y_{12}$ ( $t_1 = 0.8$ )	$y_{12}$ ( $t_1 = 1.2$ )
0.00	0.000	0.000	0.000	0.000
0.05	0.468	4.073	4.800	4.947
0.10	1.747	8.237	9.548	9.813
0.15	3.667	12.403	14.167	14.523
0.20	6.078	16.495	18.598	19.023
0.25	8.847	20.451	22.793	23.266
0.30	11.858	24.224	26.720	27.224
0.35	15.015	27.781	30.358	30.878
0.40	18.231	31.094	33.691	34.215
0.45	21.439	34.148	36.714	37.232
0.50	24.583	36.936	39.431	39.934
0.60	30.507	41.709	43.971	44.427
0.70	35.754	45.451	47.409	47.805
0.80	40.204	48.257	49.883	50.211
0.90	43.829	50.252	51.549	51.811
1.00	46.663	51.574	52.565	52.765
1.10	48.781	52.356	53.078	53.224
1.20	50.282	52.726	53.220	53.320
1.30	51.268	52.795	53.104	53.166
1.40	51.847	52.659	52.823	52.857
1.50	52.113	52.393	52.450	52.462
1.60	52.154	52.059	52.039	52.035
1.70	52.039	51.699	51.630	51.617
1.80	51.830	51.347	51.250	51.230
1.90	51.569	51.023	50.912	50.890
2.00	51.292	50.738	50.627	50.604
2.10	51.021	50.499	50.394	50.373
2.20	50.773	50.306	50.212	50.193
2.30	50.556	50.157	50.076	50.060
2.40	50.374	50.047	49.981	49.967
2.50	50.227	49.969	49.917	49.907
3.00	49.916	49.888	49.882	49.881

## 5. THE BEHAVIOUR OF THE RECOMBINATION FRACTION

The oscillatory property of the recombination fraction when the distal end of a segment varies results from the operation of interference. For the oscillation to take place the intensity of interference must reach a certain degree. Below this intensity the recombination fraction behaves monotonically, increasing steadily to its ultimate value of 50 %.

Within the present theory the nature of the interference is specified completely when the form of the distribution curve of the first point of exchange is given; that

is, the distribution  $dp = f_1(u) du$ . Consider various choices of this curve, it being supposed in each case that the total frequency and the mean are each equal to unity (i.e. that the metric is standardized). Then, the curves with the greater concentrations will represent conditions of more powerful interference than do those distributions which are more dispersed. Taking an extreme (and, of course, completely abstract example), we can let  $f_1(u)$  be the improper unit function

$$f_1(u) = \delta(u - U),$$

which is zero except at the point  $U$ . We then have

$$y(t) = 1 + (-)^{1+[t/U]},$$

and the graph of  $y(t)$  is that of a step function oscillating between values 0 and 1, the saltus points being the set

$$u = mU \quad (m = 1, 2, 3, \dots).$$

It is easy, however, to construct a smooth model, which may perhaps ultimately prove capable of fitting to linkage data, and which illuminates the kind of relation between oscillation of the recombination value and the degree of concentration of the  $f_1(u)$  curve. The function  $f_1(u) = 4ue^{-2u}$  discussed in the previous section is a confluent form of the more general function

$$f_1(u) = \frac{a+1}{a-1} \left\{ \exp \left[ -u \left( \frac{a+1}{a} \right) \right] - \exp [-u(a+1)] \right\}. \quad (49)$$

As  $a$  tends to unity this function approaches the value  $4ue^{-2u}$ .  $f_1(u)$  is normalized and has mean unity, and variance given by

$$v = \frac{a^2+1}{(a+1)^2} = 1 - \frac{1}{b} = 1 - I, \quad (50)$$

where

$$b = \frac{1}{1-v} = \frac{(a+1)^2}{2a} = \frac{1}{I}$$

and

$$a = b - 1 + \sqrt{(b^2 - 2b)},$$

so that  $2 < b < \infty$  if  $a > 1$ , while  $2 = b$  if  $a = 1$ . For any value of the parameter  $a$ , the general course of the function is similar to that of  $f_1(u) = 4ue^{-2u}$ . If the parameter  $a$  is allowed to become less than unity the curves thereby generated are identical with the family obtained for values of  $a$  greater than unity. We have

$$m(z) = 2b/(z^2 - 2bz + 2b),$$

$$M(\lambda, z) = m(z)/\{1 - \lambda m(z)\} = 2b/\{(z-b)^2 - (b^2 + 2b\lambda - 1)\}.$$

Taking  $\lambda = \pm 1$  and inverting the transforms we obtain

$$x'(t) = 1 - e^{-2bt}, \quad (51)$$

$$y'(t) = \begin{cases} \frac{2b}{\sqrt{(b^2 - 4b)}} e^{-bt} \sinh [t\sqrt{(b^2 - 4b)}] & (b > 4), \\ 8t e^{-4t} & (b = 4), \\ \frac{2b}{\sqrt{(4b - b^2)}} e^{-bt} \sin [t\sqrt{(4b - b^2)}] & (b \leq 4), \end{cases} \quad (52)$$

so that 
$$x(t) = t - \frac{1}{2b} + \frac{1}{2b} e^{-2bt}, \quad (53)$$

and 
$$y(t) = \begin{cases} \frac{1}{2} - \frac{1}{2} e^{-bt} \left( \cosh \varpi t + \frac{b}{\varpi} \sinh \varpi t \right) & (b \geq 4), \\ \frac{1}{2} - (2t + \frac{1}{2}) e^{-4t} & (b = 4), \\ \frac{1}{2} - \frac{1}{2} e^{-bt} \left\{ \cos \varpi' t + \frac{b}{\varpi'} \sin \varpi' t \right\} & (2 \leq b < 4), \\ \frac{1}{2} - \frac{1}{2} e^{-2t} (\cos 2t + \sin 2t) & (b = 2), \end{cases} \quad (54)$$

where  $\varpi = \sqrt{(b^2 - 4b)}$ ,  $\varpi' = \sqrt{(4b - b^2)}$ . For all curves of this family for which the variance is greater than or equal to the critical value  $\frac{3}{4}$ , the recombination value with the centromere rises monotonically with increasing length of segment. For variances less than the critical, interference is sufficient to produce damped oscillations which for some loci raise the value above 0.5.

The maxima and minima occur at  $t = n\pi/\sqrt{(4b - b^2)}$ ,  $n = 1, 2, \dots$ , and have the magnitudes

$$y_m = \frac{1}{2} \left\{ 1 + (-)^{n+1} \exp \frac{n\pi}{\sqrt{(4b - b^2)}} \right\}.$$

The incipient oscillation shows itself in the presence of the point of inflexion at  $t = 0.25$  on the curve

$$y(t) = \frac{1}{2} - (2t + \frac{1}{2}) e^{-4t},$$

corresponding to the critical variance.

Within this family,  $I$  the reciprocal of  $b$ , which is the same as  $1 - v$ , might be taken as a measure of the intensity of interference. More generally, remembering from § 3 that twice the deficiency  $t - x(t)$  of exchange points from the number expected on the basis of no interference is given by  $(1 - v)$ , we might adopt this quantity as an index of the amount of interference. This is not an inappropriate choice since the extreme instance,  $b = \infty$ , corresponds to unit variance and a basic distribution

$$f_1(u) = \lim_{a \rightarrow \infty} \frac{a+1}{a-1} \left\{ \exp \left[ -u \left( \frac{a+1}{a} \right) \right] - \exp [-u(a+1)] \right\} = e^{-u},$$

for which the interference vanishes.

For variance greater than unity, there is an excess of points of exchange so that a negative value of the index  $I = 1 - v$  is, naturally enough, associated with negative interference or contagion.

The index  $I$  may be shown to present itself in a second and equally natural way as a measure of the intensity of interference. As noted by Haldane (1931), the presence of positive interference will make  $V(r)$ , the sampling variance of the number of exchange points on a segment, fall short of its non-interference or Poisson value which would be merely  $\bar{r}$  or  $x_{12}$ .

In the general case we shall have for an interval  $(t_1, t_2)$  the sampling variance

$$V(r) = x_{12} - x_{12}^2 + \sum_{r=1}^{\infty} r(r-1) p_r(t_1, t_2),$$

and consequently

$$\begin{aligned} x_{12} - V(r) &= x_{12}^2 - \left[ \frac{\partial^2}{\partial \lambda^2} P(\lambda, t_1, t_2) \right]_{\lambda=1} \\ &= x_{12}^2 - 2 \int_{t_1}^{t_2} \left[ \frac{\partial}{\partial \lambda} F(\lambda, u, t_1) \right]_{\lambda=1} du. \end{aligned}$$

But 
$$F(\lambda, u, t_1) = F(\lambda, u) + (1 - \lambda) \int_0^t F(1, v) F(\lambda, u - v) dv,$$

and, therefore,

$$\begin{aligned} \left[ \frac{\partial}{\partial \lambda} F(\lambda, u, t_1) \right]_{\lambda=1} &= \left[ \frac{\partial}{\partial \lambda} F(\lambda, u) \right]_{\lambda=1} - \int_0^t F(1, v) F(1, u - v) dv \\ &= \int_0^u F(1, v) F(1, u - v) dv - \int_0^t F(1, v) F(1, u - v) dv \quad \text{by (11)} \\ &= \int_t^u F(1, v) F(1, u - v) dv. \end{aligned}$$

Hence

$$\begin{aligned} x_{12} - V(r) &= x_{12}^2 - 2 \int_{t_1}^{t_2} du \int_{t_1}^u F(1, v) F(1, u - v) dv \\ &= x_{12}^2 - 2 \int_{t_1}^{t_2} dv \int_u^{t_2} F(1, v) F(1, u - v) du \quad \text{by Dirichlet's formula.} \\ &= x_{12}^2 - 2 \int_{t_1}^{t_2} x'(v) x(t_2 - v) dv. \end{aligned}$$

Choosing  $t_1 = 0$ ,  $t_2 = t$ , then

$$\begin{aligned} x_{12} - V(r) &= 2 \int_0^t x'(v) \{x(v) - x(t - v)\} dv \\ &\sim 2 \int_0^t \{x(v) - x(t - v)\} dv \\ &\sim 2 \int_0^t \{v - (t - v - \tfrac{1}{2}I)\} dv \quad \text{as } t \rightarrow \infty \\ &= 2 \int_0^t (2v - t) dv + It \\ &= It \sim Ix_{12}. \end{aligned}$$

So that 
$$\frac{x_{12} - V(r)}{x_{12}} = 1 - \frac{V(r)}{x_{12}} \rightarrow I \quad \text{as } t \rightarrow \infty.$$

Therefore on long arms the quantity  $\{x_{12} - V(r)\}/x_{12}$ , which is a natural measure of the concentration of exchange points in excess of that to be expected on the hypothesis of a Poisson distribution, has a value approximately given by our interference index  $I = 1 - v$ .

Before proceeding to the discussion of the course of the recombination fraction in the general case we note the formulae for the general segment associated with the

function (49). The expression for  $y(t_1, t_2)$  is derived by way of (35) which supplies the most expeditious means of calculation. We have

$$x(t_1, t_2) = \left[ u + \frac{1}{2b} e^{-2bu} \right]_{t_1}^{t_2},$$

$$\begin{aligned} y(t_1, t_2) &= \frac{1}{2} - \frac{1}{2} e^{-bt_2} \left\{ e^{bt_1} \cosh \varpi D + \left( \frac{\varpi}{b} \sinh bt_1 + \frac{b}{\varpi} \cosh bt_1 \right) \sinh \varpi D \right\} \quad (b > 4, I < \frac{1}{4}) \\ &= \frac{1}{2} - (2D \cosh 4t_1 + \frac{1}{2}) e^{-4D} \quad (b = 4, I = \frac{1}{4}) \\ &= \frac{1}{2} - \frac{1}{2} e^{-bt_2} \left\{ e^{bt_1} \cos \varpi' D + \left( \frac{b}{\varpi'} \cosh bt_1 - \frac{\varpi'}{b} \sinh bt_1 \right) \sin \varpi' D \right\} \\ &\quad (2 \leq b < 4, \frac{1}{4} < I \leq \frac{1}{2}) \\ &= \frac{1}{2} - \frac{1}{2} e^{-2t_2} \{ e^{2t_1} \cos 2D + e^{-2t_1} \sin 2D \} \quad (b = 2, I = \frac{1}{2}), \end{aligned}$$

where

$$D = t_2 - t_1.$$

Figure 2 shows the density of exchange points for three cases of interest:  $b = 8, 4, 2$  corresponding to  $I = 0, \frac{1}{4}, \frac{1}{2}$ . In figure 3 we show the map distance  $x(t)$  for the same three intensities of interference, zero, critical and median ( $\frac{1}{2}$ ), while figure 4 exhibits the course of the recombination value with the centromere as the locus recedes.

upper scale—map distance from centromere,  $[x(t)]$ , in centimorgans, when  $I = \frac{1}{2}$

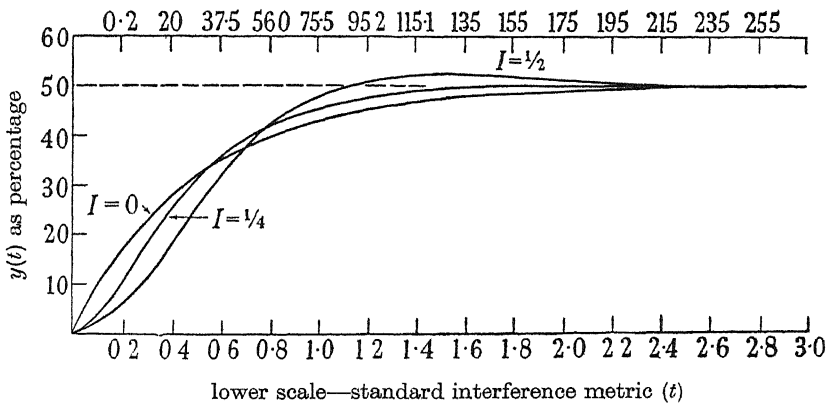


FIGURE 4. Percentage recombination with centromere for various degrees of interference.

The map distance  $x(t)$  is tabulated in table 3 for metrical values up to  $t = 2.5$ . The last figure may be in error by  $\pm 1$  for entries earlier than  $t = 1.0$ .

In the general case, the behaviour of  $x(t)$  and  $y(t)$  may be considered by extending the arguments employed in § 3.  $y(t)$  is bounded since it is equal to

$$\sum_{r=0}^{\infty} p_{2r+1}(t) \leq \sum_{r=0}^{\infty} p_r(t) = 1.$$

Hence  $\int_0^\infty e^{zt} y(t) dt$  exists in  $\text{Re } z < 0$  and equals

$$-\frac{1}{z} \int_0^\infty e^{zt} y'(t) dt = -\frac{1}{z} M(-1, z) = -\frac{1}{z} \frac{m(z)}{1+m(z)} \quad \text{by (9).}$$

So that, in  $\text{Re } z < 0$ ,

$$\int_0^\infty e^{zt} \{y(t) - \tfrac{1}{2}\} dt = \frac{1}{z} \left\{ \frac{1}{2} - \frac{m(z)}{1+m(z)} \right\} = L_2(z), \quad \text{say.}$$

It is easily verified that  $L_2(z)$  is meromorphic in the whole plane and has no poles in  $\text{Re } z \leq 0$ .

TABLE 3. MAP DISTANCE FROM CENTROMERE AS FUNCTION OF METRIC FOR VARIOUS DEGREES OF INTERFERENCE

$t$	$x(t)$ in cM ( $I = \frac{1}{2}$ )	$x(t)$ in cM ( $I = \frac{1}{4}$ )	$x(t)$ in cM ( $I = 0$ )
0.00	0.000	0.000	0
0.05	0.468	0.878	5
0.10	1.756	3.117	10
0.15	3.620	6.265	15
0.20	6.233	10.024	20
0.25	9.197	14.192	25
0.30	12.530	18.634	30
0.35	16.165	23.260	35
0.40	20.047	28.010	40
0.45	24.132	32.842	45
0.50	28.383	37.729	50
0.60	37.518	47.604	60
0.70	46.520	57.546	70
0.80	56.019	67.521	80
0.90	65.683	77.509	90
1.00	75.485	87.504	100
1.10	85.307	97.502	110
1.20	95.208	107.501	120
1.30	105.138	117.500	130
1.40	115.092	127.500	140
1.50	125.062	137.500	150
1.60	135.041	147.500	160
1.70	145.028	157.500	170
1.80	155.019	167.500	180
1.90	165.013	177.500	190
2.00	175.009	187.500	200
2.10	185.006	197.500	210
2.20	195.003	207.500	220
2.30	205.001	217.500	230
2.40	215.000	227.500	240
2.50	225.000	237.500	250

Consequently, if its poles  $\beta_r, r = 1, 2, \dots$  are simple, this function may be expanded in the form

$$L_2(z) = \sum_{r=1}^{\infty} \frac{Y_r}{\beta_r - z},$$

and, otherwise, as 
$$L_2(z) = \sum_{r=1}^{\infty} B_r^{n_r} \left\{ \frac{1}{\beta_r - z} \right\},$$

where  $B_r^{n_r}$  is a polynomial of degree  $n_r$ , if  $\beta_r$  is of order  $n_r$ .

Employing the Laplace inversion formula we have finally

$$y(t) = \frac{1}{2} + \sum_{r=1}^{\infty} Y_r^{n_r}(t) e^{-t\beta_r}, \quad (56)$$

where  $\beta_r$  is a zero of  $1 + m(z)$  of multiplicity  $n_r$  and  $Y_r^{n_r}(t)$  is a polynomial of degree  $n_r$  in  $t$ , while  $\text{Re } \beta_r > 0$  for all  $r$ . Similarly, we obtain

$$x(t) = \frac{1}{\mu_1} t - \left( 1 - \frac{\mu_2}{2\mu_1^2} \right) + \sum_{r=1}^{\infty} X_r^{m_r}(t) e^{-t\alpha_r}, \quad (57)$$

where  $X_r^{m_r}(t)$  is a polynomial of degree  $m_r$  and  $\alpha_r$  is a zero of  $1 - m(z)$  of multiplicity  $m_r$ , while  $\text{Re } \alpha_r > 0$  for all  $r$ .

It is convenient to assume that in each case the zeroes are arranged in order of increasing magnitude of the real part.

Since we have the limiting form  $y(t) \sim \frac{1}{2} + Y_1^{n_1}(t) e^{-t\beta_1}$ , the ultimate behaviour of  $y(t)$  depends on the character of the first zero  $\beta_1$  and the associated polynomial  $Y_1^{n_1}(t)$ . If  $\beta_1$  is real,  $y(t)$  is ultimately monotonic approaching the value  $\frac{1}{2}$  steadily from above or below, according to the sign of the leading term of  $Y_1^{n_1}(t)$ . But as far as our present analysis takes us, it is not possible to say that the approach must be necessarily from above, or necessarily from below. In particular, we cannot exclude the possibility that for some degrees of interference  $y(t)$  may follow one of the courses illustrated in figure 5. If  $\beta_1$  is complex  $y(t)$  must necessarily oscillate indefinitely about the value  $\frac{1}{2}$ , and in consequence recombinations greater than 50 % must be encountered indefinitely often as the segment length is increased.

We are concerned, in reality, with chromosome arms of finite length. The considerations of this paper suggest that with a sufficient length of arm (perhaps 150 to 200 cM) and intensity of interference, the recombination fraction of a locus  $P$  with the centromere may rise above 50 % as the distance from the centromere is increased.

The abstract discussion of this section, which has been included for theoretical completeness, shows, perhaps, what might be expected if nature provided sufficiently extended chromosome arms. According to the nature of the interference the value may be maintained, with or without oscillation, above 50 % as  $P$  travels out towards the terminus. On the other hand, were the arm long enough and interference more severe, several positions of  $P$  for which the recombination  $OP$  showed maximum and minimum values, lying respectively above and below the 50 % level, could be comprised within the arm length.

Where there is interference, but of low intensity, then for all lengths of arm, recombination values of all segments will lie beneath 50 %, and the recombination value of a segment is a steadily increasing function of its length.

## 6. EXPANSIONS IN POWER SERIES

If  $p(t) = 1 - \int_0^t f_1(u) du$  is analytic at the origin, and therefore has a series expansion  $p(t) = \sum_{n=0}^{\infty} \frac{p_n}{n!} t^n$  valid in some interval, finite or infinite, we can deduce recurrence relations for the coefficients in the corresponding series expansion which  $P(\lambda, t)$  will then possess.

Since the  $n$ th derivative  $p_n$  of  $p(t)$  at the origin exists, the existence of the  $n$ th derivative  $P^{(n)}(\lambda, 0) = P_n$  follows by repeated differentiation at  $t = 0$  of

$$P(\lambda, t) = 1 + (\lambda - 1) \int_0^t F(\lambda, u) du$$

and of

$$F(\lambda, t) = f_1(t) + \lambda \int_0^t F(\lambda, u) f_1(t - u) du.$$

We get

$$\left. \begin{aligned} P_1 + (\lambda - 1)p_1 &= 0, \\ P_2 + (\lambda - 1)p_2 + \lambda p_1 P_1 &= 0, \\ P_3 + (\lambda - 1)p_3 + \lambda(p_1 P_2 + p_2 P_1) &= 0, \end{aligned} \right\} \quad (58)$$

which allow the initial coefficients at least to be calculated as polynomials in  $\lambda$ . If

$$x(t) = \sum_{n=0}^{\infty} \frac{x_n}{n!} t^n, \quad y(t) = \sum_{n=0}^{\infty} \frac{y_n}{n!} t^n$$

we have, from (58), by putting  $\lambda = \pm 1$ ,

$$-x_1 = p_1, \quad -x_2 = p_2 + x_1 p_1, \quad -x_3 = p_3 + x_1 p_2 + x_2 p_1, \dots \quad (59)$$

$$\text{and} \quad -y_1 = p_1, \quad -y_2 = p_2 - y_1 p_1, \quad -y_3 = p_3 - y_2 p_1 - y_1 p_2, \dots \quad (60)$$

By a similar process the equation

$$2 \int_0^t x'(t - u) y'(u) du = x'(t) - y'(t)$$

yields

$$y_1 = x_1, \quad y_2 = x_2 - 2x_1 y_1, \quad y_3 = x_3 - 2x_2 y_1 - 2x_1 y_2. \quad (61)$$

As an example of the use of equations (58) we may take the functions employed in the discussion of the sex chromosome in the house mouse, namely,

$$\begin{aligned} p(t) &= \operatorname{sech} \frac{1}{2} \pi t = \operatorname{sech} \phi \\ f_1(t) &= \tanh \frac{1}{2} \pi t \operatorname{sech} \frac{1}{2} \pi t \frac{1}{2} \pi \\ &= \tanh \phi \operatorname{sech} \phi \frac{d\phi}{dt}, \end{aligned}$$

with  $\phi = \frac{1}{2} \pi t$ .  $p_1(t)$  comes out as  $2 \sinh \phi \log \cosh \phi / \cosh^2 \phi$ , but the higher  $p_r(t)$  cannot be evaluated in elementary functions. With the notation of § 1 and  $c = \frac{1}{2} \pi$ ,

$$p^*(\phi) = p\left(\frac{2\phi}{\pi}\right) = \operatorname{sech} \phi = \sum_{r=0}^{\infty} \frac{p_r^* \phi^r}{r!},$$



where

$$\begin{aligned} p_1^* &= p_3^* = p_5^* = \dots = 0, \\ p_0^* &= 1, \quad p_2^* = -1, \quad p_4^* = 5, \quad p_6^* = -61, \\ p_8^* &= 1385, \quad p_{10}^* = -50,521, \quad p_{12}^* = 2,702,765, \end{aligned}$$

these coefficients being Euler numbers.

The expansions of  $p_r(t)$  for  $r$  as far as  $r = 6$ , and up to  $t^{12}$ , come out as

$$\begin{aligned} p(t) &= 1 - \frac{1}{2!}\phi^2 + \frac{5}{4!}\phi^4 - \frac{61}{6!}\phi^6 + \frac{1385}{8!}\phi^8 - \frac{50,521}{10!}\phi^{10} + \frac{2,702,765}{12!}\phi^{12} \\ p_1(t) &= \frac{1}{2!}\phi^2 - \frac{6}{4!}\phi^4 + \frac{71}{6!}\phi^6 - \frac{1532}{8!}\phi^8 + \frac{53,901}{10!}\phi^{10} - \frac{2,821,683}{12!}\phi^{12} \\ p_2(t) &= \frac{1}{4!}\phi^4 - \frac{11}{6!}\phi^6 + \frac{162}{8!}\phi^8 - \frac{3638}{10!}\phi^{10} + \frac{125,028}{12!}\phi^{12} \\ p_3(t) &= \frac{1}{6!}\phi^6 - \frac{16}{8!}\phi^8 + \frac{278}{10!}\phi^{10} - \frac{6504}{12!}\phi^{12} \\ p_4(t) &= \frac{1}{8!}\phi^8 - \frac{21}{10!}\phi^{10} + \frac{419}{12!}\phi^{12} \\ p_5(t) &= \frac{1}{10!}\phi^{10} - \frac{26}{12!}\phi^{12} \\ p_6(t) &= \frac{1}{12!}\phi^{12} \end{aligned}$$

The map distance and recombination fraction are

$$\begin{aligned} x(t) &= \frac{1}{2!}\phi^2 - \frac{4}{4!}\phi^4 + \frac{52}{6!}\phi^6 - \frac{1252}{8!}\phi^8 + \frac{47,830}{10!}\phi^{10} - \frac{2,589,587}{12!}\phi^{12}, \\ y(t) &= \frac{1}{2!}\phi^2 - \frac{6}{4!}\phi^4 + \frac{72}{6!}\phi^6 - \frac{1548}{8!}\phi^8 + \frac{54,180}{10!}\phi^{10} - \frac{2,825,213}{12!}\phi^{12}. \end{aligned}$$

The initial terms of the expansion of the square of the metric in powers of the map distance are given by

$$\phi^2 = 2x + \frac{1}{3}(2x)^2 + \frac{7}{90}(2x)^3 + \frac{103}{6 \cdot 18 \cdot 35}(2x)^4 - \frac{379}{450}(2x)^5 + \frac{1,278,617}{7,484,400}(2x)^6 + \dots,$$

while, to the same order, for small map distances,  $x$  and  $y$  are related by

$$2y = 2x - \frac{1}{6}(2x)^2 - \frac{1}{18}(2x)^3 + \frac{37}{2160}(2x)^4 - \frac{547,729}{100,800}(2x)^5 + \frac{42,080,597}{342,144,000}(2x)^6.$$

It may be noted that the first two terms  $2x - \frac{1}{6}(2x)^2$  are the same as those obtained in the expansion of  $2y$  for the case when  $f_1(u) = 4ue^{-2u}$ , this agreement resulting from the closeness of this function to

$$\frac{\partial}{\partial u}(-\operatorname{sech} \tfrac{1}{2}\pi u).$$

The author is greatly indebted to Professor R. A. Fisher for suggesting study of this problem and for many helpful discussions.

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## Weismann's ring and the control of tyrosinase activity in the larva of *Calliphora erythrocephala*

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The blood and tissues of third instar *Calliphora* larvae contain a glucose dehydrogenase system which shows maximum activity at the crop-full stage and declines as pupation is approached. This decline is prevented by destruction of Weismann's ring at 3 days. It is suggested that dehydrogenase activity is the cause of the unstable reducing power of the blood and consequent inactivation of tyrosinase. The liberation of the 'pupation hormone' coincides with the complete and abrupt termination of dehydrogenase activity which leads to tyrosinase activity and hardening of the larval cuticle to form the puparium. Weismann's ring is therefore not merely active immediately before puparium formation, but exercises a regulatory influence on developmental processes during the life of the larva. Although no direct evidence is available of the existence in blowfly larvae of a juvenile hormone such as that found in *Rhodnius* by Wigglesworth, the results obtained seem consistent with the presence and later elimination of such a hormone.

### 1. INTRODUCTION

In a previous paper (Dennell 1947) an account was given of the formation of the puparium in *Sarcophaga falcifurcata*. The dihydroxyphenol responsible for hardening the cuticle of the third larval instar to form the puparium arises from the oxidation of tyrosine in the blood. As pupation is approached the blood tyrosine increases in amount, and concurrently the enzyme tyrosinase makes its appearance and increases in abundance during the latter part of larval life. In spite of the presence in the blood of both enzyme and substrate, however, no oxidation of tyrosine takes place until the pupation hormone is liberated. Then the pupal contraction occurs and the cuticle becomes hard and dark. The existence in the blood of other insects of some factor inhibiting tyrosinase activity had been noted by previous workers, notably Graubard (1933), and was fully evident in *Sarcophaga*, where it was observed

that the increase in abundance of tyrosine and tyrosinase is accompanied by a progressive fall in the oxidation-reduction potential of the blood. It was therefore suggested (Dennell 1947) that the inhibition of tyrosinase activity before pupation is due to a fall in potential below the level at which the oxidation of tyrosine is possible. Various treatments result in the release of tyrosinase activity before the normal time of pupation, the release being accompanied by a rise in potential. The possible causes of the low potential of the blood were discussed, and since the treatments resulting in rise of potential and release of tyrosinase activity bear a strong resemblance to those which in general inhibit the activity of dehydrogenases, it was speculated that it might be due to the presence in the blood of an enzyme system of this type.

This view is confirmed by the evidence presented in this paper. Not only is a dehydrogenase system truly present, but its activity is controlled by Weismann's ring which has been shown by a number of workers to exert a profound influence on the course of pupation and metamorphosis.

The work has been carried out on the third instar larva of *Calliphora erythrocephala* since considerable relevant information, which will be discussed in the text, was already available for this species and for *C. vomitoria*. The culture methods adopted were similar to those used for *Sarcophaga* (Dennell 1947). All ages quoted in the following account are those of larvae reared at 25° C.

## 2. THE DEMONSTRATION AND CHARACTERIZATION OF DEHYDROGENASE IN THE LARVA

Although the reduction of methylene blue and other dyes by the blood of *Sarcophaga* larvae under experimental conditions (Dennell 1947) may be due to their utilization as hydrogen acceptors by an enzyme of the dehydrogenase type, it is clear that in the intact larva some other acceptor must be utilized if an enzyme of this type is truly present. It was therefore desirable at the outset to study the oxygen uptake of the larval blood and tissues under various conditions. Using the Barcroft differential respirometer this has given very significant results.

It may be stated at once that the chopped tissues of larvae which have ceased to feed show a significant oxygen uptake in the presence of cyanide. Ten larvae were washed in Ringer solution, chopped and suspended in the respirometer flask in 5 ml. Ringer solution to which KCN solution had been added to give a final concentration of 0.1 M. The thermostat was maintained at 20° C. Under these conditions larvae of different ages showed oxygen uptakes varying from 6 to 36 cu. mm. in 30 min., being greatest in larvae 4 days old. That this uptake is due to enzymic oxidation carried out by the tissues is shown by the fact that it is completely inhibited by boiling the larvae for a few minutes. Since dehydrogenases are typically not inactivated by cyanide the observed oxygen uptakes may be ascribed to the activity of an enzyme of this type, and since oxygen is directly utilized as the hydrogen acceptor we may refer to the enzyme in question as an aerobic dehydrogenase.

Further evidence that the oxygen uptake of the chopped larvae is due to a dehydrogenase system is given by treating the tissues, prepared and suspended

as before, with methylene blue and phenylurethane. The addition of methylene blue in a final concentration of 0.01 % results in an initial acceleration of uptake, but this increase is not maintained, an inhibiting effect being clearly shown after 30 min. (figure 1c). The addition of phenylurethane to saturate the suspension medium causes marked depression of the uptake, corresponding to approximately 60 % after 1 hr. (figure 1d). In view of the inhibiting effects of methylene blue and narcotics on dehydrogenases generally these observations constitute confirmation of the opinion that the recorded uptakes are indeed due to the activity of such an enzyme.

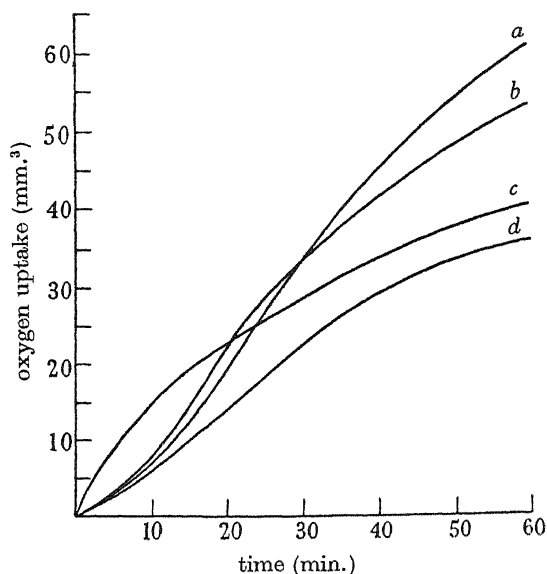


FIGURE 1. The cyanide insensitive respiration of chopped 4-day larvae of *Calliphora* under various conditions. *a*, with cyanide only; *b*, with added glucose; *c*, with methylene blue; and *d*, with phenylurethane.

In searching for the possible substrate of the enzyme the general occurrence of glucose in insect blood was borne in mind. Frew (1929) demonstrated its presence and fluctuation in amount in the blood of *Calliphora* larvae, and it seemed natural to look to it, in the first instance, as possibly constituting the naturally occurring substrate. The result, however, of adding glucose to the simple tissue preparations was disappointing. On adding glucose to a final concentration of 1 % no increased uptake is to be observed, nor is the uptake maintained for a longer period than without added glucose. Indeed, the observations point rather to a depression of the activity of the preparation (figure 1b). In view of the work of Harrison (1931), who found that a glucose dehydrogenase preparation from mammalian liver showed a marked optimum in its activity towards glucose in varying concentrations, the possibility that glucose formed the substrate sought was not immediately rejected. It seemed possible that, since the preparation consisted not only of the tissues but also of the haemolymph with its contained glucose, addition of further glucose might result in a total concentration greater than the optimum and so reduce the

oxygen uptake. It was therefore decided to attempt the preparation of a dehydrogenase extract freed from glucose by washing. Fifty 4-day larvae were washed clean, chopped, and ground with sand in cold distilled water. After several washings the ground tissues were suspended in  $M/15$   $K_2HPO_4$  and shaken gently for some time. The tissue residue was removed by centrifuging, and 2.5 ml. of the extract were added to 2.5 ml. phosphate solution in the right-hand flask of the Barcroft apparatus. KCN was added to give a final concentration of 0.1 M. The low oxygen uptake of a tissue extract prepared in this manner and shaken for 45 min. is shown in figure 2*a*. On the addition of glucose to a concentration of 1 % to a further portion of the same extract the uptake was greatly increased (figure 2*b*). It appeared probable that the slight uptake shown at *a* might be ascribed to the utilization of residual glucose in the ground tissues, and to test this preparations were made by shaking in phosphate for longer and shorter periods. The uptake shown by an extract shaken for 25 min. is seen from figure 2*c* to be considerably higher than that of the extract shaken for 45 min. Conversely the prolongation of the period of shaking to 1 hr. resulted in complete elimination of uptake. The effect of addition of glucose to the extract shaken for 25 min. is shown in figure 2*d*. From these experiments it may reasonably be concluded that not only is glucose the substrate oxidized, but that the dehydrogenase involved in its oxidation is located in the tissues and not primarily in the haemolymph which is not included in the extract.

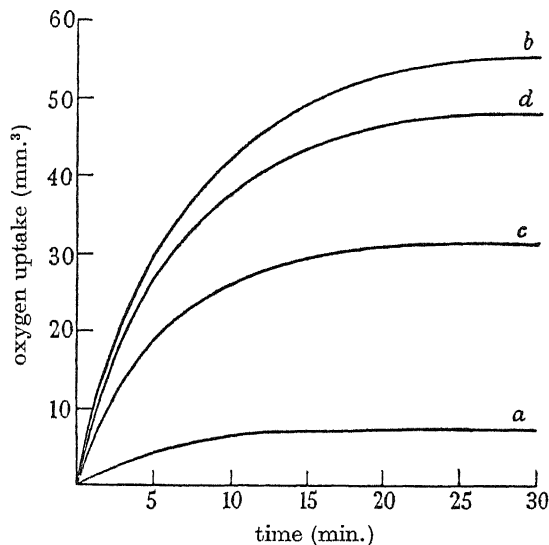


FIGURE 2. The cyanide insensitive respiration of extracts of 50 4-day larvae. Preparations *a* and *b* were extracted for 45 minutes, and *c* and *d* for 25 minutes. Preparations *a* and *c* without glucose. Glucose to a final concentration of 1 % was added to preparations *b* and *d*.

Further evidence of the occurrence of the enzyme in the tissues and only to a slight extent in the blood is given by experiments in which the oxygen uptake of chopped and washed larvae is compared with that of larvae which are chopped but not washed, so that the blood is included in the preparation. The latter preparations

consistently show a slightly higher uptake than that of the washed larvae. Additionally larvae were cut under Ringer solution and gently squeezed so that the blood was expelled. The larvae were then removed, and the blood showed only a slight uptake in the presence of cyanide. The significance of these and the preceding observations will be discussed in reviewing the work of Graubard (1933).

In view of the observations of Levenbook (1947) on fructose and the reducing value of insect blood it was of interest to repeat the observations made on the oxygen uptake of phosphate extracts, adding fructose instead of glucose. In no experiment was the addition of fructose observed to modify the residual uptake of the extract, so that the possibility that fructose may serve as substrate for the dehydrogenase studied may be excluded. This, indeed, is to be expected from the differing molecular configuration of glucose and fructose. Levenbook noted that no great amount of fructose is present in the blood of the late third instar larva of *Calliphora erythrocephala*, although it is abundant in the larva of *Gastrophilus intestinalis*.

No attempt has been made in this work to identify the oxidation product of glucose, but it is noteworthy that in experiments on phosphate extracts of larval tissues to which cyanide was added similar oxygen uptakes were recorded with and without KOH papers in the flasks of the Barcroft manometer. The course of oxidation does not therefore involve the production of  $\text{CO}_2$ . Harrison (1931) found in his study of the glucose dehydrogenase from mammalian liver that the oxidation product was gluconic acid, but his dehydrogenase showed different characteristics from that discussed here, and it does not necessarily follow that this is true of *Calliphora*.

The interpretation to be placed on these experiments is then that an aerobic glucose dehydrogenase occurs in the larva of *Calliphora* and is located principally in the tissues. Hewitt (1937) has shown that well-marked reducing conditions may be established in bacterial cultures, even when aerated, apparently as the result of dehydrogenase activity. The presence in blowfly larvae of an aerobic dehydrogenase may well therefore account for the falling oxidation-reduction potential of the blood and consequent inactivation of tyrosinase until the time of puparium formation. If this explanation is valid it is probable that fluctuations in dehydrogenase activity, related to the appearance of tyrosinase in the blood and the liberation of the pupation hormone, are to be demonstrated in growing larvae. It will be shown in the remainder of this paper that such changes not only do occur but bear an intimate relation to the activity of Weismann's ring, the gland responsible for the secretion of the pupation hormone.

### 3. CHANGES IN DEHYDROGENASE ACTIVITY DURING GROWTH OF THE LARVA

As previously stated, it was found that the oxygen uptake in the presence of cyanide was found to be greatest with larvae 4 days old, an observation amply confirmed in a study of the cyanide insensitive respiration of growing larvae up to the time of pupation.

Ten larvae were taken daily from the same batch and chopped in Ringer solution as previously described. Cyanide was added and the oxygen uptake of the

preparation observed. These experiments were repeated on numerous batches of larvae, and the results of a considerable number of determinations are expressed in figure 3*b*. It will be seen that dehydrogenase activity is first apparent at about 2 days, and rises to a maximum at 4 days. Thereafter a steady decline takes place up to the time of pupation. The maximum at 4 days, although occurring when the crop is full, cannot be due to bacterial activity in the crop contents, for it is equally apparent when observations are made on larvae from which the crop is removed, or which have been well washed after chopping. During the early part of larval life, up to about 3½ days, the larvae are still growing, and it may be objected that this alone is sufficient to account for the increase in dehydrogenase activity during this period. But on the other hand, it is clear that the fall in oxygen uptake noted after 4 days must represent a real decline in dehydrogenase activity since no change in size of the larva takes place.

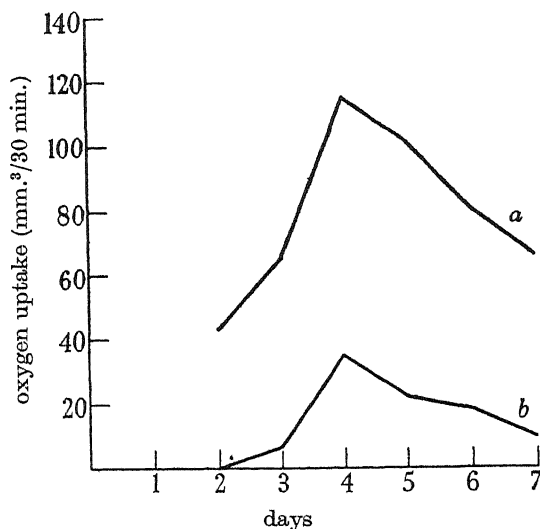


FIGURE 3. *a*, the total respiration of chopped larvae of *Calliphora*, and *b*, the cyanide insensitive respiration on successive days.

To meet this objection, daily observations were made not only of the cyanide insensitive respiration but also of the total respiration of the chopped larvae. Parallel with the rise in dehydrogenase activity the total respiration reaches a maximum at 4 days and declines towards pupation (figure 3*a*). The rise in total respiration between 2 and 4 days is accounted for by the increase in size of the larvae, and the fall after 4 days is presumably related to the more sluggish habits of the larvae after the cessation of feeding. It is now possible however to express dehydrogenase activity as percentage of the total respiration and so eliminate the effect of growth and variation in size of larvae from different batches. When this is done it is seen (figure 4) that dehydrogenase activity rises to approximately 30 % of the total respiration at 4 days, and falls to approximately 12 % not long before pupation. A true increase in dehydrogenase activity, followed by a marked decline, therefore takes place.

The figures given by Frew (1929) for the fluctuation in the glucose content of the larval blood are of immediate interest here, and are indicated by the broken curve in figure 4. At 4 days, when dehydrogenase activity and therefore glucose oxidation reach a maximum, Frew found the glucose content of the blood to be at a minimum. It is perhaps unfortunate that Frew does not expressly state the temperature at which his larvae were reared, but it is implied (p. 215) that it was 21° C and not 25° C as employed in the present work. At first sight this discrepancy appears to

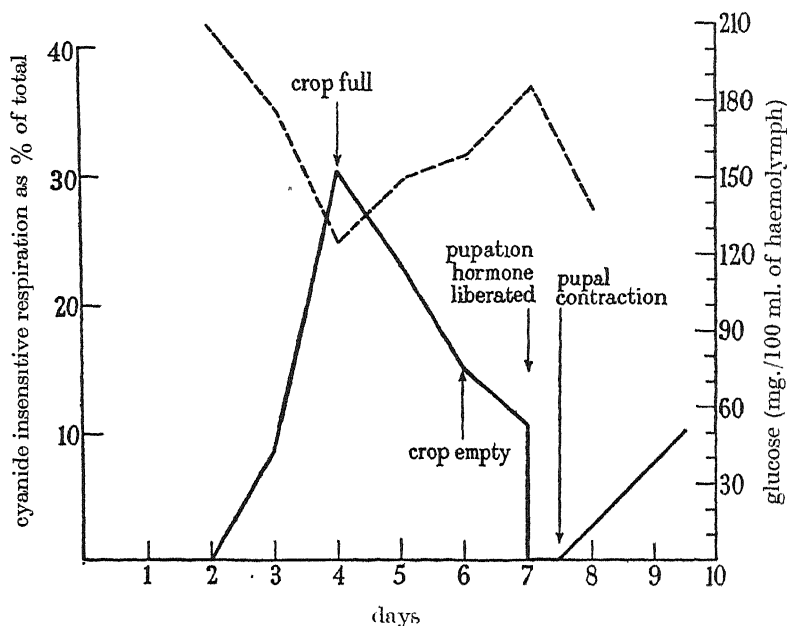


FIGURE 4. The cyanide insensitive respiration of the larvae as % of the total respiration, and the changes in glucose concentration of the blood. The figures for glucose are taken from Frew (1929).

detract somewhat from the close correspondence between the figures for glucose content and dehydrogenase activity, but it may be pointed out that in the present work observations were made at precisely daily intervals only, as those of Frew appear to have been. The maxima and minima referred to do not therefore necessarily occur precisely at 4 days, and it seems not unlikely that if observations were made at more frequent intervals of both glucose content and dehydrogenase activity in larvae reared at the same temperature close correspondence would be demonstrated. Frew remarks that 'It is probable that changes in glucose concentration may have some connection with pupation, though the connection may not be one of cause and effect'.

That the changes in dehydrogenase activity and glucose content of the larval blood are indeed intimately related to the formation of the puparium is indicated by observations on critical stage larvae in which the pupation hormone (Fraenkel 1935) has been liberated, and on white pupae. In these stages tyrosinase is active (Dennell 1947), and no dehydrogenase activity is to be expected if this is indeed the cause of tyrosinase inactivation. It is therefore important that no cyanide insensitive



respiration has been detected in larvae which have entered the critical period (as indicated by ligaturing others of the same batch, see Fraenkel 1935), nor in slightly older larvae which showed some contraction of the posterior three segments which are dragged in movement, a sign that pupation is imminent. Similarly, no uptake was observed in white pupae, but in the light brown pupae, and more markedly in older pupae, measurable uptakes were consistently recorded (figure 4). It is significant that this rise in dehydrogenase activity in the early stages of pupation is matched by a fall in the glucose content of the blood (figure 4). In contrast to these results, larvae a little before the critical period show a cyanide insensitive respiration amounting to about 12 % of the total respiration. On no occasion has an intermediate figure been recorded for larvae between the critical period and the pupal contraction.

It may therefore be postulated that when the pupation hormone is liberated at the critical period one result is the inhibition of dehydrogenase activity (figure 4). At the same time the electrode potential of the blood rises abruptly (Dennell 1947), so permitting the oxidation of tyrosine with the formation of the polyphenol responsible for the hardening of the puparium. Strong evidence of the part played by Weismann's ring in controlling the enzyme systems involved in the formation of the puparium will be given in the next sections of this paper.

#### 4. WEISMANN'S RING AND THE CONTROL OF DEHYDROGENASE ACTIVITY

Previous authors have shown clearly that Weismann's ring is intimately concerned in the hormonal control of pupation in cyclorrhaphous larvae. The existence of a hormone responsible for the onset of pupation in *Calliphora erythrocephala* was demonstrated by Fraenkel (1935), and Burt (1937) suggested that Weismann's ring, a gland dorsal to the central nerve ganglia, is the source of this hormone. Burt expressed the view that the ring constituted the homologue of the corpora allata suspected to be responsible for hormone production in other insects. Later (1938) he demonstrated decisively by experimental methods that Weismann's ring is intimately concerned with pupation in *C. vomitoria*. Non-feeding larvae 2 to 3 days before pupation from which the ring was removed, or in which it was destroyed by cauterizing, failed to pupate although they survived for a considerable time. But Burt did more than demonstrate the part played by the ring in older larvae. Turning his attention to younger larvae which had not ceased to feed, that is, aged about 3 days at 25° C, he showed that the effect of removal or destruction of the ring was not only to inhibit pupation but to cause a great extension of the feeding period. After operation the larvae continued to feed, in one instance for as long as 39 days, and died without emptying the crop preparatory to pupating.

Burt's experiments point emphatically to the activity of Weismann's ring at two distinct periods in the life of the third instar larva, and cannot be neglected here. In the present work it has been shown that at corresponding periods marked physiological changes occur in relation to the formation of the puparium, and it was therefore of critical interest to repeat the operations carried out by Burt and to determine their effect on the dehydrogenase activity of the larvae.

Since it was considered inadvisable for the present purpose to open the larvae they were cauterized as described by Burt (1938) except that they were not anaesthetized before the operation. The effects of the operation on the behaviour of the larvae were precisely as described by Burt, but further than this, study of the oxygen uptake of preparations of the operated larvae in the presence and absence of cyanide revealed profound modifications of the fluctuations in dehydrogenase activity shown by normal larvae. Initially the respiratory characteristics of unoperated larvae were observed, and a considerable number of larvae were then cauterized for later observation. Larvae cauterized 24 hours before pupation (figure 5*c'*), that is, before the critical period at which the pupation hormone is liberated, show no sudden inhibition of dehydrogenase activity such as occurs in normal larvae at the critical period (figure 4). In the operated larvae dehydrogenase activity continues unchecked for some time after the normal period of pupation. A somewhat similar result is given when Weismann's ring is cauterized in larvae  $3\frac{1}{2}$  days old (figure 5*a'*). Dehydrogenase activity once more remains approximately constant, continuing at the level characteristic of larvae of this age until well after the normal time of pupation. Larvae cauterized at  $4\frac{1}{2}$  days show a different result (figure 5*b'*). Dehydrogenase activity falls with increasing age of the larvae which, unlike those cauterized at  $3\frac{1}{2}$  days, cease to feed, but again does not show inhibition at the critical period. Different results are therefore produced by cauterization immediately before and immediately after the period when dehydrogenase activity of the normal larva reaches its height.

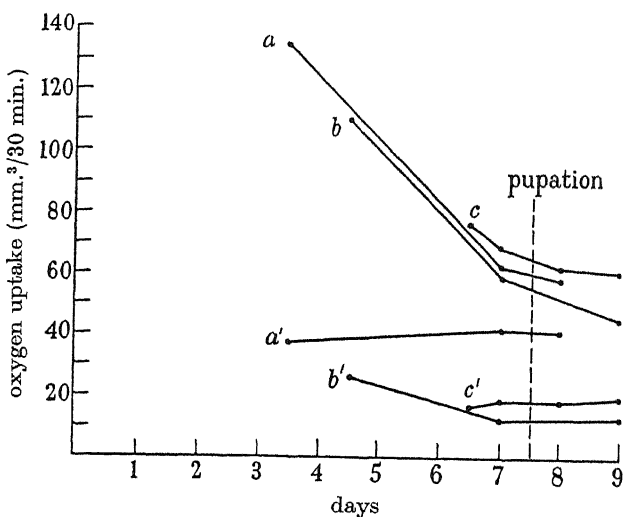


FIGURE 5. The effects on respiration of cauterizing Weismann's ring at different times. *a*, *b* and *c*, total respiration; *a'*, *b'*, and *c'*, cyanide insensitive respiration. The time of pupation of normal larvae is indicated at  $7\frac{1}{2}$  days.

The effect of cauterization on total respiration is illustrated in figure 5*a*, *b* and *c*. A progressive fall takes place similar to that occurring in normal larvae. As a result the dehydrogenase activity of larvae cauterized at  $3\frac{1}{2}$  days, expressed as percentage of total respiration, shows a marked increase from 28 to 66 % in the experiment

illustrated. This is in marked contrast to the fall occurring in normal larvae (figure 4), but it should be pointed out that the operated larvae are often more sluggish than the normal so that the fall in total respiration is probably exaggerated.

The simplest explanation of these results would be that not only is Weismann's ring active at two distinct periods in larval life, as Burt (1938) found, but at these periods it exercises control over the glucose dehydrogenase system described. Observations on normal and cauterized larvae indicate that this control is not exercised in the same way at the critical periods of 4 and 7 days. It seems that the hormone which may be presumed to be liberated at 4 days checks the production of further dehydrogenase by the tissues, with the result that a progressive decline in activity of this enzyme follows. At the critical period of 7 days the pupation hormone completely inhibits residual dehydrogenase activity, so setting in train the events leading to the hardening of the larval cuticle as the puparium. A less simple explanation, however, arises from the work of Wigglesworth (1934, 1936, 1940, 1947, 1948) and will be discussed later.

##### 5. WEISMANN'S RING AND THE CONTROL OF TYROSINASE PRODUCTION

It has been shown (Dennell 1947) that in *Sarcophaga* the blood of larvae from the crop-full stage onwards darkens on exposure to the air for some minutes, indicating the presence of both tyrosine and tyrosinase. The delay in darkening seems to be due to the progressive disappearance of the inhibiting conditions which prevent darkening of the blood in intact larvae. Further, and separately from this observation, tyrosine and the oenocytoid-like cells suspected to secrete tyrosinase were shown to increase in the blood from the crop-full stage onwards. Since in the present work Weismann's ring has been shown to control dehydrogenase activity and therefore tyrosinase activity, it was necessary to discover whether it exerts a direct effect on tyrosinase production.

Experiments have therefore been carried out on the darkening of exposed blood of *Calliphora* larvae cauterized before and after the crop-full stage. The blood of larvae cauterized at 3 days is found to darken on exposure on filter paper more strongly than that of unoperated larvae when examined at 6½ days, that is, before the time of the critical period. No doubt is left of the greater potency of the tyrosinase system in the operated larvae, in spite of the greater dehydrogenase activity already referred to. A similar though less marked difference between the blood of operated and control larvae is obtained by cauterizing at 4½ days, and a still smaller difference when older larvae are cauterized. It is to be noted that the darkening of the blood of the operated larvae, although more intense than that of control larvae, takes place more slowly, as might be expected from the fact that operation at about 3 days results in greater dehydrogenase activity. This darkening of the blood of operated larvae is inhibited by cyanide, as is that of control larvae when examined before the critical period. It is therefore not due to the non-enzymic oxidation of a phenol already present in the blood.

These results indicate that while activity of Weismann's ring at 4 days does not appear to initiate tyrosinase production, the ring exerts a controlling

influence from this time onwards, and may be regarded as maintaining the balance between potential tyrosinase activity and the inhibiting dehydrogenase system.

## 6. DISCUSSION

The view that tyrosinase activity in the blood of the larval blowfly is held in check by the low oxidation-reduction potential produced by the activity of a dehydrogenase system finds support from a number of sources. The occurrence of such a system is entirely adequate to explain the low potential of the blood found before pupation, and it is to be particularly emphasized that the disappearance of dehydrogenase activity shortly before pupation, at the critical period when the pupation hormone is liberated, is accompanied by a rapid rise in potential (Dennell 1947). The reduction of dyes by the blood may be ascribed to the action of a dehydrogenase, and the darkening of exposed blood only after an interval to the fact that not until the reducing power of the blood is eliminated, either as a result of destruction of dehydrogenase or disappearance of its substrate by oxidation, is oxidation of tyrosine possible. The effect of dilution of the blood in accelerating darkening may perhaps be due to the reduction of glucose concentration below the optimum for dehydrogenase activity, although the possibility of a direct effect on the rate of destruction of the enzyme cannot be excluded. And further the treatments effective in inducing the premature darkening, though not contraction, of intact larvae due to tyrosinase activity show a striking correspondence with those which in general inhibit dehydrogenases.

The results obtained by Graubard (1933) are in agreement with the conclusions reached during this work. Graubard found that some inhibiting factor or substance is present in *Drosophila* larvae, and is in some way less accessibly situated than is tyrosinase. Extracts made by grinding the larvae with sand showed greater inhibition of tyrosinase than those prepared without sand, a result completely in accordance with the observation here that dehydrogenase is present to only a slight extent in the blood, being mainly found in the tissues. The further observation of Graubard that extraction with chloroform water results in a high yield of tyrosinase agrees with the fact that chloroform is an effective inhibitor of dehydrogenases.

The work of Kuwana (1937) is also significant here. In this work it was found that the reducing power of silkworm blood shows considerable diminution, though not complete disappearance, on exposure. This diminution is accompanied by melanosis, and Kuwana was of the opinion that the unstable reducing power of the blood might be due to the presence of a phenol which on exposure becomes oxidized to a coloured compound. But in the light of the present work it is understandable that on exposure dehydrogenase activity of the blood may diminish, so causing a loss of reducing power. Similarly the observations of Levenbook (1947) are important in indicating the instability of insect blood. The blood of the third instar larva of *Gastrophilus* contains a considerable amount of fructose which diminishes when the blood is allowed to stand, suggesting that here perhaps a fructose dehydrogenase may be operative. It may well be, indeed it is probable, that in different insects different dehydrogenase systems may serve as inhibitors of tyrosinase

activity, just as within the general plan of the mechanism of phenolic hardening of the cuticle different oxidation products of tyrosine appear to be involved (Pryor, Russell & Todd 1947).

It may be concluded, then, that in blowfly larvae tyrosinase activity is inhibited by the agency of a dehydrogenase system as was suggested as a result of work on *Sarcophaga* (Dennell 1947). Neither the presence of ascorbic acid as in plants, nor of a phenol as suggested in the silkworm by Kuwana (1937), is adequate to explain the observed inhibition of tyrosinase. The possibility that the apparent liberation of tyrosinase activity in blowfly larvae at the time of puparium formation is actually the result of the production of tyrosinase from its precursor protyrosinase under the action of a lipidic activator, as occurs in the eggs of *Melanoplus* (Bodine 1945), has not been confirmed. It should be emphasized, however, that while it is convenient to speak of tyrosinase inhibition this is not strictly correct. Tyrosinase is not actually inactivated, and the appearance of inactivation arises from the fact that at the low potential obtaining in the blood the oxidation of tyrosine is not possible.

The significance of the control of dehydrogenase activity by Weismann's ring must now be considered. During the past 15 years it has become increasingly apparent that moulting and metamorphosis in insects takes place under the hormonal control exercised by the components of a neuroglandular complex situated in the head. The components of this system are the corpora cardiaca and allata, and the pars intercerebralis of the brain. Weismann's ring of the cyclorrhaphous larva of Diptera contains the histological elements of the corpus cardiacum and corpus allatum and is responsible for the control of growth, moulting, and pupation (see Scharrer & Scharrer 1944). Following the work of Fraenkel (1935) considerable evidence has accumulated pointing to the production by Weismann's ring of a hormone specifically causing pupation, and Becker & Plagge (1939) have claimed to have isolated this hormone and have enumerated some of its properties. Becker & Plagge showed that older larvae are more susceptible than younger ones to the action of the hormone. They ligatured larvae of *Calliphora* before the critical period so that the posterior portions were unable to receive the hormone and therefore did not pupate. Injection of the blood of critical stage larvae, however, in which the pupation hormone was present, into these posterior portions caused pupation. Receptors in which the anterior end had pupated 14 to 24 hours after ligaturing were more sensitive to the action of the hormone than those in which pupation of the anterior end had occurred 72 to 96 hours after ligaturing. Fraenkel (1935) had previously found similar differences in larvae ligatured at different periods before pupation. These results are not in conflict with the observations on the fluctuations in dehydrogenase activity made in the present work. Reference to figure 4 of this paper shows that the larvae ligatured by Becker & Plagge a little before the critical period would show less dehydrogenase activity than those ligatured earlier. Inhibition of dehydrogenase by the pupation hormone may therefore be expected to be most complete in these larvae, with the result that they form the most sensitive receptors. But it must be remembered that the blood of critical stage larvae used as donors contains not only the hormone but also some

polyphenol arising from its action, so that it is difficult in these experiments to distinguish between the effects of the hormone and the direct action of the polyphenol. It is to be noted that later Becker (1941) adopted the view that the material previously isolated (Becker & Plagge 1939) was not the hormone but some chemical to which the integument would respond after activation by the hormone.

So far it has been presumed that the hormone liberated at the critical period is indeed specifically a pupation or metamorphosis-inducing hormone, but it seems possible that it is no more than a general developmental and therefore moult-inducing hormone which evokes pupation and metamorphosis only in virtue of the conditions obtaining at the time it is liberated. This view arises from a consideration of the theory of metamorphosis put forward by Wigglesworth (1934, 1936, 1940, 1947, 1948) as the outcome of his work on *Rhodnius*, and supported by embryological and histological findings (Henson 1946). In *Rhodnius* two hormones are responsible for control of the course of development. One is secreted in the dorsum of the brain and causes growth and development with the production of imaginal characters. It is referred to by Henson (1946) as a general developmental hormone, not merely as a moulting hormone as Wigglesworth described it, and this procedure will be followed here. In the nymphal instars the activity of this hormone is modified by a second, the juvenile, hormone secreted by the corpus allatum, so that the adult form is not assumed. The assumption of the imaginal condition is therefore due to the developmental hormone exerting its full effect in the 5th instar nymph in the absence of the juvenile hormone. Wigglesworth (1947, 1948) gives reasons for believing that in the 5th nymph the corpus allatum not only does not secrete juvenile hormone, but actively removes from the blood any traces that remain. He goes on to suggest that it may be the elimination of juvenile hormone in Diptera which gives the impression of a 'metamorphosis-promoting hormone'.

Although there is as yet no direct evidence of the production and elimination of a juvenile hormone in cyclorrhaphous Diptera Wigglesworth's view has much to recommend it, and the evidence in its favour may be reviewed here.

Burt (1938) studied the effects of removal or destruction of Weismann's ring in young third instar larvae of *Calliphora*, and found that removal of the ring at 3 days causes arrest of development. The imaginal buds cease to grow, and the larva continues to feed. These results may be interpreted by supposing that during the first 3 or 4 days of larval life juvenile hormone is present, but is later eliminated by the ring gland. Extirpation of the ring at 3 days therefore prevents the disappearance of the hormone, so that the imaginal buds remain in a retarded or juvenile condition. Further, in the present work it has been found that up to 4 days dehydrogenase activity of the larva rises and thereafter falls, and that cauterizing the ring at 3 days prevents this fall. If we are justified in regarding the production of dehydrogenase as a juvenile character, since it inhibits tyrosinase activity and therefore opposes the production of the puparium, then in accordance with the interpretation placed on Burt's results it is found that conditions promoting the juvenile state prevail in the early part of larval life. Perhaps the production of dehydrogenase by the tissues is stimulated by juvenile hormone, and the decline in dehydrogenase activity which occurs after 4 days follows the disappearance of this hormone.

In *Rhodnius* the corpus allatum appears to produce the juvenile hormone when exposed to the stimulus provided by the secretion of the developmental hormone, so that the juvenile hormone eliminated by the corpus allatum in the fifth stage nymph is that remaining from the previous moult. In the fifth stage nymph the developmental hormone, secreted on the stimulus of feeding, results in the absence of juvenile hormone in mitosis of the epidermal cells in 5 days, the appearance of new cuticle in 18 days, and moulting after 28 days (Wigglesworth 1940). Although the imaginal cuticle is still not fully formed it is hardened immediately after ecdysis, and further production of endocuticle takes place for at least 3 weeks (Wigglesworth & Gillett 1936). In *Calliphora* the developmental hormone responsible for the initiation of the production of the cuticle of the third instar larva is liberated at the end of second instar life, and as in *Rhodnius* cuticle formation is incomplete when ecdysis takes place. Growth of endocuticle takes place after moulting, and may even continue after the pupal contraction as in *Sarcophaga* (Dennell 1947). But hardening of the cuticle does not take place on ecdysis, as it does in *Rhodnius*, it is delayed until the end of the third larval stadium when puparium formation takes place. The developmental hormone liberated at the end of this stadium is in no way related to the secretion of the third instar cuticle or to the possible early presence of juvenile hormone, but is that which initiates the production of the prepupal cuticle. If juvenile hormone is indeed present in *Calliphora* at the beginning of the third instar it must, if Wigglesworth's views are applicable, have been secreted by the ring gland in response to the stimulus provided by the liberation of the developmental hormone of the second instar. Secretion of the cuticle of the early third instar larva therefore proceeds in the presence of juvenile hormone and therefore conforms to the larval pattern.

From this comparison emerges the essential difference in the time of hardening of the cuticle in the adult *Rhodnius* and the puparium of *Calliphora*. In *Rhodnius* hardening occurs at the beginning of the stadium, but in *Calliphora* the hardening of the third instar cuticle to form the puparium is delayed so that the process of puparium formation is overlapped by the developmental cycle which involves the production of the cuticle of the next instar. To this may be ascribed the function of the developmental hormone liberated at the end of third instar life in inducing hardening of the puparium. Whereas in *Rhodnius* the developmental hormone does not, apparently, provide the stimulus to hardening of the cuticle, in *Calliphora* the hormone is liberated at precisely the time when the components of the enzyme mechanism responsible for hardening the cuticle have been assembled. It appears that by inhibiting dehydrogenase and so releasing tyrosinase activity the developmental hormone has assumed the additional function of stimulating puparium formation, whereas in insects generally the stimulus causing hardening of the cuticle remains obscure (Wigglesworth 1939).

If we adopt the view held by Wigglesworth (1947, 1948) and Henson (1946) the critical feature of metamorphosis is the withdrawal of the modifying influence of the juvenile hormone so that growth and differentiation are directed towards the development of imaginal characteristics. The decline in dehydrogenase activity after 4 days noted in the present work, and the observation of Burt (1938) that the

destruction of Weismann's ring at 3 days arrests the growth of imaginal buds, suggests that the elimination of juvenile hormone may begin in *Calliphora* at about 3 days before pupation. It is noteworthy that after this time the crop begins to empty, a process accompanied in *Sarcophaga* by the appearance of tyrosine and tyrosinase in the blood and the production of polyphenol oxidase by the epidermal cells (Dennell 1947). These are, of course, preparations for puparium formation and it is evident that the crop-full condition is a decisive period in the developmental sequence. Additional evidence of this is given by the observations of Drummond (1939), who ligatured *Sarcophaga* larvae of different ages for a short period and then freed them. Larvae subjected to temporary ligature before the critical period underwent pupation in both halves, indicating, as Fraenkel (1935) had pointed out for *Calliphora*, that the stimulus to pupation is not nervous since the effect of the temporary ligature is to interrupt connexion between the central and peripheral parts of the nervous system. But when the operation was repeated on younger larvae many remained alive without pupating after the normal larvae from the same batch had pupated. Drummond therefore suggested that since the ring gland was present in these larvae which failed to pupate the stimulus causing liberation of the pupation hormone cannot have been received. As a result of a number of experiments on larvae of different ages he concluded that in *Sarcophaga* the interval between stimulus and pupation is about three days. It is clear, however, that Drummond's results may equally well be explained by the suggestion that at about 3 days before pupation stimulation of the ring gland takes place and is followed by the elimination of juvenile hormone. If the stimulus is not received juvenile hormone persists and dehydrogenase activity does not decline, so that the developmental hormone liberated later is unable to effect complete inhibition of this enzyme and puparium formation therefore does not take place. In connexion with Drummond's observations it should be noted that Burt (1938) found that severing the nerves between Weismann's ring and the brain in *Calliphora* larvae 4 days before pupation had the same effect as removal or destruction of the ring.

The work of Bodenstein (1943, 1944) on *Drosophila* seems also to point to the existence of a decisive phase in larval life. Bodenstein showed that not only growth but differentiation is controlled by the ring gland, and came to the conclusion that the same hormone is involved in both these processes. He states (1944) that whether growth or differentiation takes place in organ disks depends on a definite relationship between hormone level and organ competence. Young organ disks of third instar larvae are able to respond to hormone stimulation with growth, but as they become older this capacity decreases, while their capacity to respond with differentiation increases. Although Bodenstein explained his results by reference to hormone level and tissue competence, they may possibly be better explained in terms of the elimination of juvenile hormone.

Throughout this paper, as a matter of convenience, the terms 'puparium formation' and 'pupation' have been used as synonymous. It is evident however that the term pupation should be restricted to the production of the pupa, whereas puparium formation is the earlier hardening of the larval cuticle. Both puparium formation and pupation result from the onset of metamorphosis, which on the



views suggested here must be regarded as initiated in the larva as early as the end of the feeding period. The term 'pupation hormone' applied to the hormone liberated at the critical period at the end of the third instar therefore appears to be misleading. In conclusion, it seems that Weismann's ring is to be regarded as not merely active immediately prior to pupation, but as influencing the whole course of third instar larval life.

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#### APPENDIX

After the completion of this paper my attention was drawn by Dr V. B. Wigglesworth to the paper by Danneel (1946) recording the presence of a dehydrase in insects. Since this paper is as yet inaccessible to me I am unable to comment on its significance in the light of the present work.

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# A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and of nutrients

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A novel technique for determining the total and the meristematic number of cells in a root meristem is described; it involves macerating the tissue in a known volume of fluid, and subsequently determining the appropriate number of cells in an aliquot on a haemocytometer slide. Root tips are cultured in batches on sintered glass disks with different nutrient media and at four temperatures. Samples of four roots are taken from each culture at intervals of 12 hr., and with each sample the average total number, the average meristematic number of cells, and the average length of each root are determined. From these primary data rates of division and indices of extension are calculated.

It is shown that no division occurs in the absence of sugar, and the rate of division is increased by providing inorganic salts with the sugar, and still further increased by supplying yeast extract. With each nutrient the rate of division is higher at 15° than at 25° C, but lower at 5° than at 15° C. No extension occurs in the absence of sugar, and the greatest extension is given with sugar and inorganic salts. Yeast extract in the form and concentration used in these experiments tends to depress extension.

The results of the main body of experiments are discussed along with others showing the effects of single mineral deficiencies. It is suggested tentatively that in these experiments the level in the cells of certain products of carbohydrate degradation determine the rate of cell division, and that in cell extension the process depends to some extent on a synthesis of protein which requires a supply of sugar and of certain inorganic salts.

## INTRODUCTION

This investigation provides an analysis of the effects of certain experimental treatments on the processes of cell division and cell extension as they occur in the isolated meristem of the root, and it thus furnishes the data for determining the relative contribution that each process may make to the total growth of the root. The investigation is a development from the pioneer work of Burström (1941, 1947) in this field; it is based, however, on entirely novel techniques and is designed to supplement the earlier work not only by extending it to a new research object but also by providing more extensive data, particularly with regard to nutritional effects in cell division. For the purposes of the inquiry a technique for determining the number of cells in a tissue has been developed which may have many further applications.

The tissue studied has been the root apex of *Cucurbita pepo* and the experimental design has involved culturing short root tips in batches of thirty or forty, with three nutrient media and at four temperatures. For each culture, at intervals of 12 hr., samples of four roots are taken, and on each sample the average length of the root, the average total number of cells per root and the average total number of non-vacuolated cells per root are determined. From these primary data two derived

sets of values are calculated, which are measures respectively of rates of cell division and of the degree of cell extension. Serial values have thus been assembled which show the effects with time of the experimental variables on (a) the overall length of the root, (b) the rate of cell division, and (c) the degree of cell extension.

## EXPERIMENTAL METHOD

### *Cultural techniques*

The root tips are excised from young seedlings of *Cucurbita pepo* which have been germinated, without their testas, on damp filter paper at 25°C. The removal of the testas ensures a uniform root development. When the radicles are 1.5 to 2 cm. long they are ready for use; they are detached from the seeds and their tips are inserted into holes of approximately their own diameter in a metal plate 1.6 mm. in thickness which rests on another sheet of glass or metal. When all the roots are in position the tips are severed by passing a razor across the surface of the plate. The plate is then lifted from its base and the isolated tips, each 1.6 mm. in length, are shaken out of the perforations and transferred immediately to the culture vessel.

The excised roots are cultured on sintered glass disks, 3 in. in diameter, contained in petri dishes into which culture fluid is poured to a level just below the upper rim of the sintered glass. Thirty or forty tips are placed on each disk and the cultures are incubated in the dark.

### *Experimental treatments*

Three basic nutrient media have been used. White (1932) has shown that extensive root systems are developed from root tips when a carbohydrate, a suitable mixture of inorganic salts, and yeast extract are supplied. These three types of nutrients have therefore been used here, the particular media incorporating them being 2 % sucrose, an inorganic salt mixture dissolved in 2 % sucrose, and a yeast extract dissolved in 2 % sucrose containing the inorganic salts. Preliminary observations indicated that, with respect to the carbohydrate, 2 % sucrose gives the most satisfactory results. The inorganic salt mixture was based on that recommended by White and consisted of 100 mg. calcium nitrate, 25 mg. magnesium sulphate, 35 mg. potassium carbonate, 25 mg. potassium dihydrogen phosphate and 1.25 mg. ferric sulphate, all per litre of fluid. The yeast extract was supplied in the form of a commercial preparation (Yeastrel) at the rate of 1 mg. per 100 ml. of fluid.

Each nutrient medium was used at four temperatures, namely, 25°, 20°, 15° and 5°C. Treatment at the two higher temperatures was effected in two constant temperature rooms maintained at 25° and 20°C respectively; at 15°C in an unheated cellar in which the temperature fluctuated between 14° and 16°C during the time of the experiments; and at 5°C in the main chamber of a large refrigerator.

The experimental period in all series was restricted to 3 days in order to avoid the necessity of maintaining sterile conditions in the cultures. The use of sterile conditions would necessarily have restricted the scope of the inquiry, since the techniques used require large quantities of material. Within the period of 3 days with appropriate media the roots grow appreciably and normally remain free of

fungal or bacterial attack. Moreover, with the culture technique used, the media do not develop the heavy cloudiness of massive bacterial contamination.

At intervals of 12 hr., samples, each of four roots, were taken at random and, after the length of each root had been measured against a ruler, the total number of cells and the number of non-vacuolated cells in the sample were determined.

### *Cell counting technique*

The principle involved in this technique is simple. The roots are first macerated in a known volume of fluid in such a way that the tissue is dismembered either into individual cells or into groups of cells sufficiently small for the number in them to be exactly counted. The density of the cell suspension or of a particular component in it is then determined by the application of a normal haemocytometer technique.

The suitability for the present purpose of a number of macerating agents including those recommended by Tupper-Carey & Priestley (1923) was examined, but only one, namely 5 % chromic acid, gave satisfactory results. The technique finally developed as a standard procedure involves immersing the roots in 2 ml. of this solution at room temperature for 12 hr., and then shaking the whole mass vigorously by hand. This treatment yields a turbid suspension in which normally there are no clumps visible to the naked eye. If occasional clumps still remain they are dispersed by pressing them gently against the side of the vessel with a glass rod. The period of 12 hr. for treatment with chromic acid is not critical, and it may be extended to 24 hr. but, with more prolonged exposure to the acid, cell walls are attacked and the cells do not survive the shaking necessary to disperse them through the fluid.

When maceration is complete a sample of the suspension is withdrawn and a drop introduced below the cover-slip of a haemocytometer slide. During this process two precautions must be observed. Some of the cells tend to fall rapidly in the fluid, and the removal of the sample must therefore be preceded by a vigorous agitation of the suspension. For the same reason, during the transfer of the material to the haemocytometer slide, it is desirable to keep the pipette as nearly horizontal as possible.

A haemocytometer slide was used, with a depth of 0.2 mm. between the cover-slip and the grid. The slide is placed on the mechanical stage of a binocular microscope equipped with a  $\frac{2}{3}$  in. objective and  $\times 10$  eyepieces. The grid of the slide covers an area of 16 mm.<sup>2</sup> and is divided into 256 squares in 16 horizontal rows. The number of cells in each row is counted and recorded separately, the numbers for the separate rows being finally added together to give the number of cells in 3.2 mm.<sup>3</sup> of fluid. From this the number of cells in the original suspension is calculated, and from this again the average number in each root is determined.

With each sample taken from the suspension the area over the grid is scanned twice. On the first occasion all the cells, and on the second the non-vacuolated cells only, are counted. In the normal procedure two samples are taken from each suspension, and each value given in the next section is based on the mean of duplicate determinations.

The method outlined above for determining cell numbers is relatively rapid, since each count occupies only about 10 min. Moreover it is reasonably accurate. Replicate haemocytometer readings from the same suspension usually agree within about 5 %; four readings from the same suspension for instance gave the following values: 482, 473, 466, 515.

At the same time the technique yields values for total numbers that are of the same order as estimates made by other methods. The total number of cells in a cylindrical segment of the root has been estimated by the method used by Sinnott (1942) with other tissues; the average volumes of cells in the cortex and the pith have been determined and these have been divided into the total volumes of the respective tissues. Two determinations by this method gave 69,240 and 65,177, and two by the maceration technique 75,888 and 70,625. The agreement between the two sets of determinations is thus reasonably close, and shows that there is little, if any, destruction of cells during the maceration procedure.

#### *Errors involved in the cultural technique*

The variation in the number of cells in the root tips immediately after excision is not greater than 8 %. Haemocytometer readings with six different groups of four tips each gave the following mean values: 416, 450, 437, 441, 418, 431. After growth in the cultures has continued for some time, the differences between corresponding samples taken from duplicate cultures is of course greater but does not exceed 10 %. Determinations of total numbers of cells made with samples taken from duplicate cultures at 25°C, and with three nutrient conditions are given in table 1, the sets of values marked I in this table being also included in table 3.

TABLE 1. DUPLICATE SERIAL DETERMINATIONS OF TOTAL NUMBER OF CELLS PER ROOT WITH THREE NUTRIENT MEDIA AT 25°C.: 2 % SUCROSE (*S*), INORGANIC SALTS IN 2 % SUCROSE (*MS*), AND WITH YEAST EXTRACT ADDED TO THE *MS* MEDIUM (*YMS*)

hr.	cell numbers $\times 10^{-3}$					
	<i>S</i>		<i>MS</i>		<i>YMS</i>	
	I	II	I	II	I	II
12	66.2	69.2	81.9	80.3	115.0	114.0
24	71.5	76.5	84.3	86.1	128.0	119.2
36	75.9	77.5	94.9	100.9	135.6	124.2
48	79.5	75.4	96.5	91.8	126.1	126.5
60	80.6	75.9	99.0	95.1	125.4	123.2
72	80.4	82.6	105.3	102.9	134.2	133.1

The differences between successive values of table 1 are a measure of the number of cells formed in the root during the corresponding intervals. In this connexion, however, it may be noted that a small systematic error is involved which is shown by the values obtained with water only given in table 2. In this series in the absence of cell division the total number of cells in the root decreases from about 67,000 to 61,000. This decrease has been observed on several occasions and is undoubtedly due to cells being shed from the surface of the root. A comparable

loss of cells is probably involved in all series, but proportionately is likely to be less than the 10 % observed with water only, and has been ignored in the consideration of the relevant data presented below.

*Values derived from primary data*

From the primary data two derivative sets of values have been calculated—'rates of cell division', and 'indices of extension'.

From successive values the total number of cells formed during any interval may be determined. The increment in the total cell number is not, however, even a relative measure of the rate of cell division since during any interval the number of meristematic cells may change. The assumptions have been made that division occurs primarily in non-vacuolated cells which are all potential meristematic cells, and the rate of division during the 12 hr. interval has been calculated by dividing the total cell increment by the average number of non-vacuolated cells in the root during the interval. As shown below, the number of non-vacuolated cells in the experimental conditions of this investigation usually decreases with time and the average number has been taken as the arithmetic mean between the numbers at the beginning and end of the interval. Thus, if  $T_1$  and  $m_1$  are respectively the total and meristematic numbers of cells at the beginning, and  $T_2$  and  $m_2$  the corresponding numbers at the end of the interval, then the 'rate of division' is given by  $T_2 - T_1 / (m_1 + m_2) / 2$  and represents the number of divisions per meristematic cell per 12 hr.

The reliance to be placed upon the value for the 'rate of division' depends of course on the accuracy with which the total cell number is determined, on the error involved in the determination of the number of non-vacuolated cells, and on the justification for the assumptions on which the calculation is based. The errors of the total cell count have been considered above. The errors involved in the non-vacuolated cell count are not easy to determine since it is difficult to identify all non-vacuolated cells in what is virtually fixed material. During treatment with chromic acid newly formed vacuoles may disappear. The error from this source, however, will tend to give an under- rather than an over-estimate for the rate of division. Priestley (1929) has shown that divisions may occur in vacuolated cells and if these are extensive a rate of division based on non-vacuolated cells only would be too high. Evidence of such divisions was sought for but was not forthcoming; divisions of this kind cannot therefore have been frequent. The further assumption that all non-vacuolated cells are meristematic cells lacks any experimental evidence; nevertheless, it is justified by the observation that mitotic figures are distributed over the whole apical region of non-vacuolated cells.

The 'index of extension' is a ratio between root length and the number of vacuolated cells at the corresponding stage of growth. Since non-vacuolated cells are restricted to about 0.5 mm. from the tip, and since the number of these has been deducted from the total number of cells, the ratio is a comparative measure of the average lengths of vacuolated cells. The ratio embodies of course all the uncertainties involved in the determination of the total and the non-vacuolated cell numbers, and as a measure of average length it is valid only if the number

of cells in the cross-section of the root does not vary, which the evidence available suggests is the case. Large differences in thickness between roots from different treatments have not been observed, and the slight differences noted have always accompanied corresponding variations in length, and may therefore be attributed to effects on the size of the individual cells.

The ratio between root length and the number of vacuolated cells is undoubtedly a measure of the average cell length but as a measure of extension growth its adequacy depends upon how much of the root fragment is occupied by extending cells, and upon the number of extending cells that are added from the extreme apex during the period of observation. If extending cells occupy the whole fragment (apart from the extreme apex) and if their number does not increase then the index is a valid measure of extension growth. If the fragment includes older cells which have ceased to extend, the index loses validity, and if a relatively large number of vacuolating cells is added to the fragment during each interval of the experimental period then the course of extension in the original cells may be obscured by that in the cells formed during the experiment. It has been shown that a segment cut out of the root, extending from 1.5 to 3.0 mm. from the tip, increases in length when placed on suitable nutrient media. This observation indicates that all the cells within 1.5 mm. from the tip are still in an early stage of extension. Moreover, as shown below, after 24 to 36 hr. there is virtually little increase in the number of vacuolated cells.

#### EXPERIMENTAL RESULTS AND THEIR INTERPRETATION

The main body of results presented here have been obtained with three basic media, but in certain connexions it is necessary to compare these with two control series using respectively water only and an inorganic salt solution only. The results of these two control series are set out in table 2 from which it is clear that there is no increase either in the lengths of the fragments or in the total numbers of cells if water or a solution of salts without other nutrients is used. It is however significant that in both series the number of meristematic cells decreases sharply with time.

TABLE 2. EFFECTS OF DISTILLED WATER ALONE AND OF SOLUTIONS OF INORGANIC SALTS ONLY, AT 25°C, ON TOTAL CELL NUMBER  $T$ , ON THE NUMBER OF MERISTEMATIC CELLS  $C$ , AND ON OVERALL LENGTH OF ROOTS AT SUCCESSIVE STAGES OF DEVELOPMENT  $L$

hr.	Cell numbers $\times 10^{-3}$					
	distilled water			inorganic salt solution		
	$T$	$C$	$L$ (mm.)	$T$	$C$	$L$ (mm.)
0	67.3	34.0	1.6	67.3	34.0	1.6
12	62.5	21.5	1.8	77.0	23.2	2.0
24	63.9	15.3	2.0	67.0	14.0	2.5
36	59.7	12.4	2.0	69.3	13.3	2.6
48	61.2	7.3	2.0	67.1	16.7	2.1
60	61.2	3.1	2.0	65.3	6.1	2.1
72	61.2	0.0	2.0	63.0	3.0	2.1

The results obtained with the three basic nutrient media at the four temperatures may next be considered in relation to (a) cell division, (b) cell extension, and (c) the overall increase in length of the roots.

Cell division

The change in the total number of cells with time in each experimental series is shown in table 3, while the data given in this table for 25°C, together with the relevant data from table 2, also obtained at 25°C, are displayed graphically in figure 1.

TABLE 3. TOTAL NUMBER OF CELLS PER ROOT AT FOUR TEMPERATURES WITH 2 % SUCROSE (*S*), INORGANIC SALTS IN 2 % SUCROSE (*MS*), AND WITH YEAST EXTRACT ADDED TO THE *MS* MEDIUM (*YMS*)

cell numbers $\times 10^{-3}$												
hr.	25°C			20°C			15°C			5°C		
	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>
0	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3
12	66.2	81.9	115.0	95.5	102.1	122.3	126.3	117.9	130.4	89.8	89.9	97.5
24	71.5	84.3	128.0	110.1	94.5	121.7	127.2	137.9	160.5	92.9	83.8	96.4
36	75.9	94.9	135.6	97.4	116.0	132.5	121.9	136.3	148.1	87.2	90.0	97.0
48	79.5	96.5	126.1	104.3	109.8	128.8	123.8	132.0	149.5	89.3	88.5	95.0
60	80.6	99.0	125.4	109.6	109.8	135.5	125.9	137.9	152.0	91.0	87.0	98.1
72	80.4	105.3	134.2	105.7	117.0	144.0	119.7	128.0	144.2	89.9	86.9	95.0

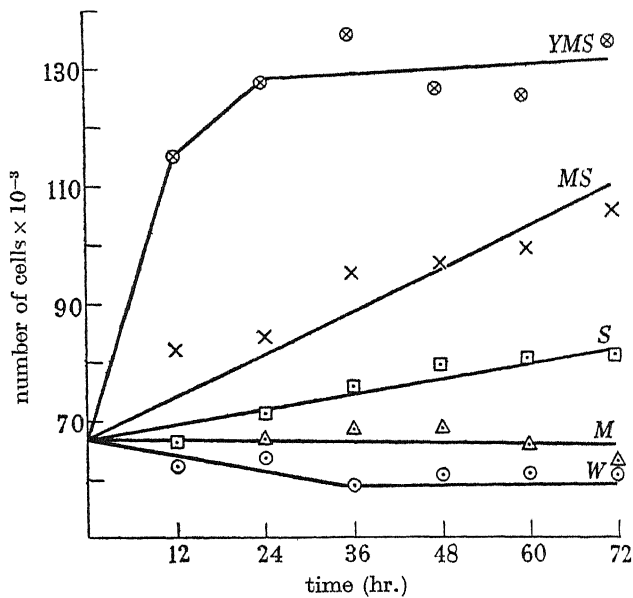


FIGURE 1. Total number of cells at 25°C, with water *W*, morganic salt solution *M*, 2 % sucrose *S*, inorganic salts in 2 % sucrose *MS*, and with yeast extract added to *MS* medium *YMS*.

In all series of table 3, except for the sugar *S* and for the sugar and salts *MS* cultures at 25°C, there is a sharp increase in the number of cells during the first



24 hr., but the values for the final 48 hr. do not immediately indicate any consistent increase. Accordingly the results of table 3 have been examined statistically, the analysis being based on the values for 24 to 72 hr. inclusive, the group for each temperature being treated separately, and the total variance in each case being assigned to time, treatment and error. At each temperature the differences due to treatment are highly significant, but only with the group at 25°C is the increase with time significant and then only at the 5 % level. The significant increase with time at 25°C is clearly due entirely to the series with sugar *S* and with sugar and salts *MS*; with yeast extract added to the other two nutrients *YMS* the position is similar to all other series, since after a sharp initial increase there is little or no consistent change during the final 48 hr.

The data of table 3 show that the number of cells formed is increased by the addition of salts to sugar and further increased by the addition of yeast extract; but the data of table 2 and figure 1 show that the salt effect is not given in the absence of sugar, and when sugar is not provided no cells are formed at all. Burström (1941) has also shown with isolated roots of wheat that no cells are formed in the absence of sugar, and Addicott (1940) that the number of mitotic figures in the apices of isolated pea roots is increased when they are provided with vitamin B<sub>1</sub> and nicotinic acid, which in these experiments are probably being added with the yeast extract.

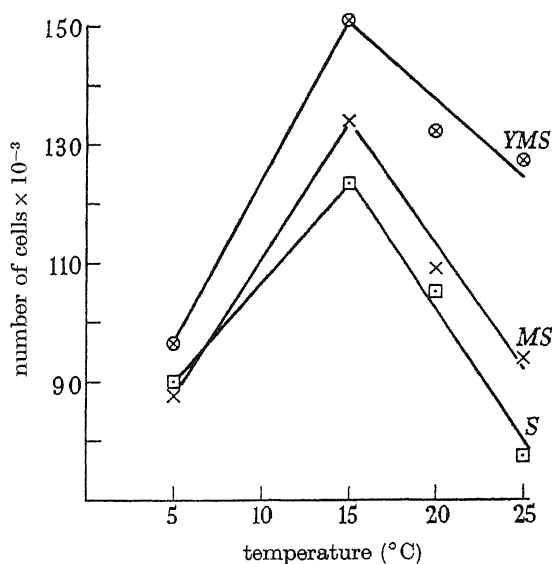


FIGURE 2. Averages of total cell numbers for period 24 to 72 hr., for each nutrient medium plotted against temperature. Symbols as in figure 1.

The temperature effect is emphasized by the curves of figure 2, in which the means based on the values for 24 to 72 hr. inclusive of each group with the same nutrient are plotted against temperature. Whatever the nutrient the number of cells formed at 15° is greater than at 5°C; but at 20° the number is lower than at 15°C, and at 25°C it is still lower. These results may be compared with those

obtained by Burström (1941) with different methods and using wheat roots. This worker found that the number of cells formed per unit time increased with increase in temperature from 7° to 26°C. The interpretation of the differences between these two sets of results is discussed below.

The differences in the number of cells formed in unit time may of course be due to corresponding differences in the number of cells available for division, to differences in the rates of division per meristematic cell or to a combination of both circumstances. The numbers of non-vacuolated cells in the roots of different series at successive stages are shown in table 4, and while it is evident from these data that the number of meristematic cells tends to decrease progressively throughout the experimental period, and particularly rapidly after 24 hr., nevertheless the differences at the end of the first 12 and first 24 hr. are not directly related to the differences in the total number of cells formed in these intervals.

TABLE 4. NUMBER OF NON-VACUOLATED CELLS WITH DIFFERENT NUTRIENT MEDIA AND AT DIFFERENT TEMPERATURES AT SUCCESSIVE STAGES OF GROWTH. SYMBOLS AS IN TABLE 3

Cell number $\times 10^{-3}$												
hr.	25°C			20°C			15°C			5°C		
	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>
0	34.0	34.0	34.0	34.0	34.0	34.0	34.0	34.0	34.0	34.0	34.0	34.0
12	21.6	30.1	36.0	49.3	42.8	53.5	58.4	27.9	37.2	33.0	27.3	30.8
24	17.3	19.0	31.1	35.0	44.4	53.9	22.1	22.0	30.6	31.5	25.6	25.7
36	12.7	15.6	18.9	27.7	35.4	32.7	18.8	13.2	12.8	32.5	26.4	32.5
48	8.9	16.8	10.2	29.9	43.7	32.8	11.8	16.1	12.9	34.7	23.1	33.0
60	6.1	18.2	8.1	17.0	19.0	19.2	10.1	11.8	12.0	30.2	26.0	29.8
72	1.4	17.2	1.9	14.0	22.7	21.4	8.4	4.8	15.1	28.1	22.2	31.5

TABLE 5. RATE OF DIVISION (NUMBER OF DIVISIONS PER MERISTEMATIC CELL PER 12 HR.) WITH DIFFERENT NUTRIENT MEDIA AND DIFFERENT TEMPERATURES AT SUCCESSIVE STAGES OF GROWTH. SYMBOLS AS IN TABLE 3

hr.	25°C			20°C			15°C			5°C		
	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>
0 to 12	0	0.45	1.36	0.68	0.91	1.26	1.28	1.63	1.77	0.67	0.74	0.93
12 to 24	0.28	0.12	0.38	0.33	—	—	—	0.80	0.88	0.09	—	—
24 to 36	0.36	0.59	—	—	—	—	—	—	—	—	—	—
36 to 48	0.33	0.10	—	—	—	—	—	—	—	—	—	—
48 to 60	0.14	0.15	—	—	—	—	—	—	—	—	—	—
60 to 72	0.0	0.34	—	—	—	—	—	—	—	—	—	—

The rates of cell division calculated from the primary data of tables 3 and 4 are given in table 5. Where the primary data do not show any consistent increase after 24 hr. no rates have been calculated, and they have not been calculated for the 12 to 24 hr. period when the second value of table 3 is not greater than the first. The data of table 5 show emphatically that the differences in the number of cells formed during the first 24 hr. are due primarily to corresponding differences in the rates of division. During the first 12 hr. at all temperatures the rate is in-

creased by adding inorganic salts to sugar and still further increased by adding yeast extract. With regard to the effect of temperature, with one exception, the rate is higher at 20° than it is at 25°C, and higher again at 15° than at 20°C. All values at 5° are lower than the corresponding values at 15°C. For the two series at 25°C in which division continues throughout the experimental period the rate (expressed as the number of divisions per meristematic cell per 12 hr.) with sugar and salts is 0.29, and with sugar alone it is 0.19.

The rates of division given in table 5 may also be expressed in terms of the time occupied by a single cycle of division in each meristematic cell. In this form the rates for the first 12 hr. (taking for *S* at 25°C half the value for 12 to 24 hr.) are:

temperature (°C)	rate (in hr.)		
	<i>S</i>	<i>MS</i>	<i>YMS</i>
25	83.3	26.7	8.8
20	18.2	13.2	9.5
15	9.4	7.4	6.8
5	17.9	17.6	12.9

### Cell extension

The tips at the time of excision contain about 34,000 vacuolated cells, and to these others are added in the course of growth, as vacuolation proceeds in the mass of non-vacuolated cells. The whole group of vacuolated cells increases in size and the extension established in individual cells with different experimental treatments may be estimated from the indices of extension given in table 6, which are calculated from the data of tables 3, 4, and 7.

TABLE 6. INDICES OF EXTENSION (ROOT LENGTH (MM.)  $\times 10^3$ /NUMBER OF VACUOLATED CELLS). EXPERIMENTAL TREATMENTS AND SYMBOLS AS IN TABLE 3

hr.	25°C			20°C			15°C			5°C		
	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>
12	45	58	33	43	42	36	24	22	17	32	29	27
24	92	107	62	53	60	59	19	28	15	29	31	25
36	85	113	60	72	68	46	27	28	27	33	28	28
48	99	100	78	70	98	73	35	41	29	33	28	29
60	81	136	77	67	77	60	47	38	39	30	30	26
72	88	159	68	72	74	65	49	45	47	29	28	28

As indicated above (p. 115), the significance of successive indices depends on the rate at which the number of vacuolated cells may increase. During the final 36 hr of the experimental period the proportionate increase in the number of vacuolated cells for all treatments at 25°C is about 10 %, and at 20°C it is about 20 %; at 15° and 5° C there is little or no increase. Thus at all temperatures during the final 36 hr. the value of the index is determined by the behaviour of cells that had vacuolated before this period, and large changes in the value of the index during the period therefore reflect changes in the relative sizes of individual cells throughout the mass of extending cells.

At 25°C with sugar *S* and with yeast salts and sugar *YSM* there is evidently no extension after 36 hr., but with salts and sugar *MS* extension continues throughout the final 36 hr., and at the end of the experimental period the greatest extension has been made by the series provided with sugar and salts, and the least by that in which yeast extract has been added to the medium. At 20°C there is little or no extension with any treatment during the final 36 hr., and at this temperature the average cell extension is again greatest with salts and sugar and least with yeast extract added to the *MS* medium. At 15°C extension continues throughout the final 36 hr., although at this temperature differences due to nutrient treatment are very small. At 5°C there is no extension during the final 36 hr. and again little or no nutrient effect.

Thus, at temperatures above 15°C, as with cell numbers, the addition of salts to sugar increases extension, but unlike the position with cell numbers the addition of yeast extract to the medium (at least in the form and concentration used here) decreases cell extension. The data of table 2, however, indicate that, as with cell numbers, cell extension only occurs in the presence of sugar, and the salt effect is not observed in the absence of sugar.

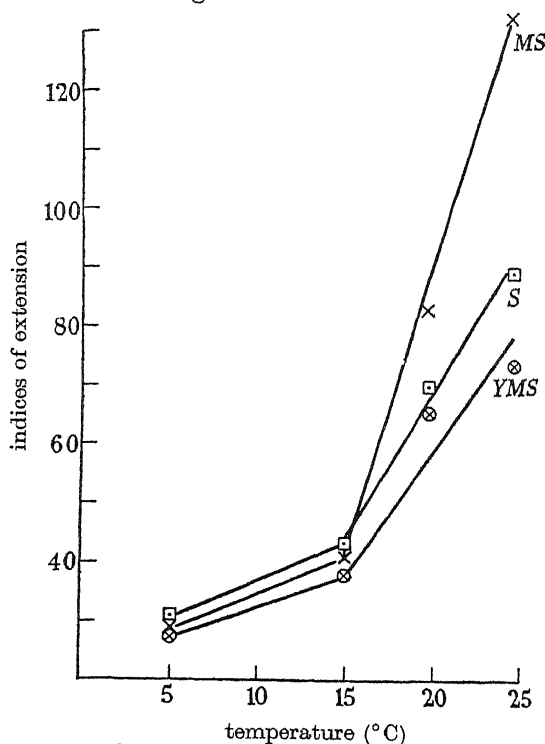


FIGURE 3. Averages of indices of extension for period 36 to 72 hr. for each nutrient medium plotted against temperature. Symbols as in figure 1.

The effect of temperature on the relative extension at the end of the experimental period is shown by the curves of figure 3 in which the means for the values over the final 36 hr. for each nutrient treatment are plotted against temperature. Clearly with all nutrient treatments the final extension increases with increasing

temperature. It may be noted that, while the values plotted in figure 3 are a crude measure of the rate of extension, they suggest, within their limitation, that in all treatments the temperature coefficient between 15° and 25°C may be greater than 2, and between 5° and 15°C it may be less than 2. The high temperature coefficient for the upper temperature range suggests strongly that within it extension is controlled by enzyme catalyzed reactions.

Burström (1941) also finds from observations on the epidermal cells of roots that sugar is necessary for extension; but the results of his observations on the effect of temperature differ in some respects from those recorded above. The present series of results agree with those of Burström in that the rate of extension increases with increasing temperature, but they do not show as his do that the final length of the cell decreases with increasing temperature. Extension in our material is complete in all series at 20°C at the end of the experimental period, but the final indices are lower than the corresponding figures at 25°C. Since Burström's results agree with ours (in connexions already considered and with others discussed below) with regard to nutrient effects, but differ with regard to temperature effects both in cell division and cell extension, the differences may be attributed to the use of the roots of different species.

#### *Overall increase in length of the root*

In the course of the experiments the length of each root of each sample is measured, and an average value for each sampling occasion is determined. The serial averages for each experiment are given in table 7.

TABLE 7. LENGTH (MM.) OF ROOTS WITH DIFFERENT NUTRIENT MEDIA AND AT DIFFERENT TEMPERATURES. SYMBOLS AS IN TABLE 3

hr.	25°C			20°C			15°C			5°C		
	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>	<i>S</i>	<i>MS</i>	<i>YMS</i>
0	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
12	2.0	3.0	2.6	2.0	2.5	2.5	1.6	2.0	1.6	1.8	1.8	1.8
24	5.0	7.0	6.0	4.0	3.0	4.0	2.0	3.0	2.0	1.8	1.8	1.8
36	5.4	9.0	7.0	5.0	5.5	4.6	2.8	3.4	3.6	1.8	1.8	1.8
48	7.0	8.0	9.0	5.2	6.5	7.0	3.9	4.8	4.0	1.8	1.8	1.8
60	6.0	11.0	9.0	6.2	7.0	7.0	5.4	4.8	5.5	1.8	1.8	1.8
72	7.0	14.0	9.0	6.6	7.0	7.0	5.5	5.5	6.0	1.8	1.8	1.8

The results of table 7 may be interpreted by reference to the effects discussed above of the experimental treatments on cell extension and cell division. It is evident that whatever the nutrient treatment the growth in length of the root tends to decrease with decreasing temperature. Between 25° and 15°C with decrease in temperature there is an increase in number of cells (figure 2), and therefore within this range the decrease in overall length is due to a progressive decrease in cell extension (figure 3). On the other hand, at 72 hr. the average length of the cells at 15°C is approximately one-third greater than at 5°C whereas the root length is approximately three times as great.

At 20°, 15°, and 5°C nutrient treatment has no effect on change in length of the root with time; but at 25°C the differences due to nutrient treatment are well

defined. At 25°C the sugar and salts *MS* medium gives a greater overall length than the sugar, salts and yeast extract medium *YMS*. The *MS* medium, however, while giving a smaller number of cells gives a greater cell extension, and it is to this condition that the greater overall length must be attributed. Again at 25°C the *YMS* medium gives a larger root than the simple sugar medium *S*. The more complex medium, however, while giving the shorter average cell length gives the larger number of cells, and the greater overall length must therefore be due to this second condition.

#### DISCUSSION

The results presented above show that in most series division ceases after the first 24 hr. and they indicate that the process is limited during the final 48 hr. by some condition that is not limiting during the first 24 hr. The results also show that at least in the first phase division is promoted by adding sugars, salts and yeast extract to the medium, and by decreasing the temperature from 25° to 15°C. The interpretation of these results must necessarily remain somewhat tentative until the work can be extended to other material in which division continues uniformly for longer periods than the 24 hr. to which in most series it has been restricted in these experiments. Nevertheless it is possible to analyze the situation further even at the present stage of the inquiry; but before doing so the probable cause for the cessation of division after 24 hr. in certain series may be considered.

In the conditions of these experiments division after 24 hr. may be limited either (1) by the activity of micro-organisms, or (2) by the exhaustion of a nutrient reserve present in the tips at the time of excision.

In these experiments sterile conditions were not maintained and although there were no visible signs of massive bacterial or fungal infection in the cultures, the possibility nevertheless remains that such infection may have been sufficiently extensive to affect the cultural conditions especially in the immediate vicinity of the roots on the surface of the disks. In that case the observed effect of a reduction in temperature from 25° to 15°C might be due to a consequent reduction in the intensity of contamination. It is significant, however, that the temperature effect is given with sugar alone, as well as with the more complex media, and when neither salts nor yeast extract are given with the sugar it is highly improbable that there could be any considerable contamination. Secondly, the growth of micro-organisms is likely to have been most intense with the sugar, salts and yeast extract medium, and the temperature effect with cell division if it is due to an effect on the degree of contamination might be expected to be relatively greatest with the most complex medium and least with sugar alone. In fact, as shown by the data of figure 2, the converse is the case. The greater relative effect with sugar alone is also shown by the means of the appropriate sets of results of table 3. At 25° and 15°C respectively for sugar alone, the means are 74.5 and 115.7, and for sugar, salts and yeast extract 118.1 and 121.7. Thirdly, if the growth of micro-organisms is an important factor then the effects should be particularly intense when salts are added to sugar at 25°C. The results of table 3,

however, show that in these conditions division continues throughout the experimental period.

It is probable therefore that the second of the possibilities outlined above is the cause of the cessation of division after 24 hr. The exhaustion of a nutrient supply in the tips may, however, be due either to the consumption of the reserve in the formation of new cells or to outward diffusion of the substance concerned from the tips into the medium. If the reserve is dissipated into the medium then the rate of dispersal may be higher at 25° than at 15°C, giving a greater increase in the number of cells at the lower, than at the higher temperature. It has been shown experimentally that a nutrient dispersal from the tips does occur. Fluid in which tips have been immersed for 24 hr. has been used as the medium for a second set of tips, and in these an enhanced increase in the number of cells has been observed. Nevertheless, it is probable that different rates of dispersal at different temperatures are not primarily involved in the temperature effect for the following reasons. At the same temperature the dispersal of a reserve through outward diffusion should always occupy the same time, and cell increase should, therefore, be limited to a constant period. In fact it is not since at 25°C with sugar and salts and with sugar the increase is continuous throughout the experiment, but with yeast extract, sugar and salts the increase is confined to the first 24 hr. Secondly, during the first 24 hr. with sugar at 25°C the number of cells formed is 4000, and at 15°C it is about 60,000. If the number of cells formed is proportional to the level of the reserve in the tip, then the position would require a fifteen-fold greater outflow at the higher than at the lower temperature.

Thus the evidence suggests that the cessation of division is due to the exhaustion of a nutrient reserve through consumption in the formation of new cells and that it is the absence of this nutrient from the medium that limits division during the final 48 hr. Further, during the first 24 hr. the temperature and nutrient effects observed may be interpreted as direct effects on the process of division.

The results emphasize the central importance of a supply of carbohydrate for both cell division and cell extension. The root tips when they are excised evidently do not carry any carbohydrate reserves and unless an external supply is provided both processes cease.

With regard to cell division, it is clear that although the carbohydrate may be essential as a source of metabolic energy, other aspects of the metabolism of the substance are involved. Inorganic salts accelerate division, but only in the presence of carbohydrate. This no doubt indicates an effect of the salts on the utilization of carbohydrates. The stimulation due to yeast extract may be interpreted in the same sense especially as it can be observed in the absence of salts (relevant data shown in table 8). An increase in the rate of cell division, however, also accompanies reduction in temperature from 25° to 15°C, but again only in the presence of carbohydrate. Measurements have been made of the change in numbers of cells with time at 15°C when water only is supplied, and the results are essentially the same as those obtained with water at 25°C. Thus carbohydrates are also essential to the temperature effect, but whereas it is probable that salts and yeast extract accelerate the metabolism of sugar, it is clearly highly improbable that a reduction

in temperature can have this effect. Almost certainly the rate of metabolism is lower at 15° than it is at 25°C.

These results may be interpreted in terms of a dependence of the rate of cell division on a supply of intermediate substances arising from carbohydrate degradation, which schematically may be represented as involving a change from sugar *A* to a complex of substances *B*, leading to a second complex *C*. The rate of cell division, it may be suggested, depends on the level of *B*. Further, the rate of the *A* to *B* reaction depends on the supply of salts and yeast extract, and the rate of the *B* to *C* reaction is depressed to a greater extent by a reduction in temperature from 25° to 15°C than is the rate of the *A* to *B* reaction. Thus the level of *B* tends to rise when either salts and yeast extract are supplied, or when the temperature is depressed.

The level of the complex *B* no doubt determines the rate of division by providing metabolites which are synthesized into substances which are required in the process of division. The nature of the complex *B* is unknown, but the interpretation proposed above is consistent with the results of other preliminary experiments in which the effects of certain deficiencies in the inorganic salt mixture have been determined. In these experiments the anions of calcium and magnesium salts have been replaced by the corresponding chlorides, and the relevant results are shown in table 8. It is evident that neither sulphate nor nitrate deficiency has any immediate effect, and the only anion deficiency that immediately depresses cell division is that of phosphate. In the absence of phosphate there is little or no increase in cell number. Similar results have been recorded by Burström (1947) with low nutrient levels of phosphate and nitrate. The importance of the phosphate supply may of course be referable to the need of this material for the synthesis of nuclear components many of which contain phosphorus, but it is equally probable that it accelerates the utilization of carbohydrates, and thus the accumulation of the hypothetical complex *B*. It may be noticed that the results given in table 8 for the phosphate-deficient medium indicate less division than with sugar alone, although sugar is supplied along with the deficient salt mixture. Evidently, certain of the components of the salt mixture tend to depress division. That this is so is confirmed by the results obtained with certain other deficiencies. The iron-deficient medium, for instance, gives an abnormally rapid initial increase in cell numbers. The significance of the stimulation with this deficiency cannot be determined from the present series of data.

With cell extension the necessity of sugar for the appearance of the inorganic salt effect, and the high value of the temperature coefficient for the rate of this process between 25° and 15°C, both indicate that sugar is involved in metabolic processes on which extension depends. The sugar may provide a source of metabolic energy, it may be required for a synthesis of cellulose that is necessary for extension, or it may accumulate in the vacuole and promote extension by increasing the osmotic pressure of the cell sap. The results with deficient media, however, suggest that at least part of the sugar effect must be attributed to the necessity of sugar for the synthesis of amino-acids which are essential for extension. Burström (1947) has already shown that extension is reduced by low levels of nitrate in the nutrient



and the relevant indices of extension given in table 8 show that in the absence of nitrates and sulphates extension is depressed. Nitrates it may be suggested are required for the synthesis of amino-acids in general and sulphates for the synthesis of the sulphur-containing acids in particular. Clearly the synthesis cannot occur if products of sugar degradation are not at the same time available. These results, therefore, suggest that extension depends on a synthesis of proteins. Further, Blank & Frey-Wyssling (1944) have already shown with the hypanthium of *Oenothera*, and Brown (1946) with the embryo of barley, that a synthesis of protein does occur during cell extension. It may be noted from table 8 that phosphate deficiency does not reduce extension. Burström (1947) has also noted a similar effect.

TABLE 8. INDICES OF EXTENSION  $E$  AND TOTAL CELL NUMBERS  $T$  AT 25°C WITH YEAST EXTRACT IN 2 % SUCROSE  $YS$ , INORGANIC SALTS IN 2 % SUCROSE  $MS$ , AND THE FOLLOWING DEFICIENCIES IN  $MS$  MEDIUM: PHOSPHATE  $MS-P$ , IRON  $MS-I$ , SULPHATE  $MS-S$ , NITRATE  $MS-N$

Values under $T$ are cell numbers $\times 10^{-3}$						
hr.	$MS$		$YS$		$MS-P$	
	$E$	$T$	$E$	$T$	$E$	$T$
0	48	67.3	48	67.3	48	67.3
12	58	81.9	38	96.1	108	66.8
24	107	84.3	52	119.0	118	64.3
36	113	94.9	66	113.9	158	75.3
48	100	96.5	64	111.3	158	66.1
60	136	99.0	66	113.9	268	64.2
72	159	105.3	69	111.0	208	62.0

hr.	$MS-I$		$MS-S$		$MS-N$	
	$E$	$T$	$E$	$T$	$E$	$T$
0	48	67.3	48	67.3	48	67.3
12	44	104.3	94	80.1	67	99.8
24	63	115.8	82	98.1	77	100.0
36	103	111.8	80	96.2	81	95.2
48	124	111.8	78	97.9	87	97.0
60	—	—	82	99.0	85	100.3
72	91	112.1	87	107.1	79	103.2

The results obtained with sugar resemble in many respects those observed by Schneider (1938) in the coleoptile, but he found that heteroauxin much enhanced the sugar effect. The heteroauxin effect in the root will be discussed in a later paper.

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# The mechanics of crossing-over

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A theoretical treatment of the frequency distribution of chiasmata along a chromosome is developed, based on Darlington's postulate of relating crossing-over to chromosome coiling. Each chromatid is assumed to be an elastic thread subject to torsion and longitudinal stress, which is liable to break (but not yield) at a definite breaking load. It is supposed further that when an interchange occurs after a break, the consequential relief of torsional strain is confined to a small region round the location of the break, resulting in a lowering of the probability of a further break. The assumption about the effect of a break is formulated in a suitable way and an expression for the distribution function is thence derived. It is shown that in the limit of no interference this distribution goes over to the Poisson type, in conformity with Haldane's conclusions. Expressions are calculated for the mean and the standard deviation of the distribution; the former gives the relation between the mean chiasma frequency and chromosome-length.

When homologous chromosomes pair in meiosis, there is a mutual shift or interchange of segments. This 'crossing-over' is a biological event of fundamental importance, and many theories have been offered which attempt to explain the precise mechanism involved during this process. According to an attractive theory proposed by Darlington (1935) crossing-over is related to chromosome coiling. Two homologous chromosomes which are unwinding their relic coils pair in such a way that their internal and relational coils reach equilibrium. As a result of division of each chromosome into two thinner chromatids the strain of coiling is supposed to lead to a break in one of the chromatids. The consequent readjustment of forces results in a non-identical chromatid breaking at the same level. The broken ends of one chromatid are then very likely to meet those of the other and this produces crossing-over. The local reduction of coiling stresses involves a lowering of the possibility of a further break in that region, resulting in interference.

The theory of Darlington outlined above is satisfactory as affording a qualitative explanation of the various aspects of the event. But a quantitative approach will require a more precise specification of the nature of a chromosome in relation to its torsional and breaking properties. To this end it is supposed that each chromatid may be treated as an elastic thread subject to torsion and longitudinal stress (load). The assumption is made that for each thread there is a definite load (breaking load) at which it breaks, but does not yield. In an earlier contribution (Srinath 1946) it was shown how, on the basis of the elastic theory, one may derive a proof that when one of the chromatids breaks the other also experiences a break at the same level. This paper embodies a further elaboration of the elastic theory, and on the basis of suitable assumptions, a distribution function is derived for multiple crossing-over, taking explicit account of the effect of interference. It will appear that in the

absence of interference, the distribution reduces to the Poisson distribution, and this is in agreement with Haldane's conclusions (1931).

As indicated above, each chromatid is supposed to be an elastic thread with torsion, and that the two threads are in equilibrium under the torsional and longitudinal stresses. When an interchange occurs after a break, it is supposed that the consequent relief of torsional strain is confined to a small length  $\epsilon/2$  on either side of the break. It is postulated that when a break has occurred, a further break cannot occur within a length  $\epsilon/2$  on either side of the location of the break. Further, it is also assumed that  $\epsilon$  is small in comparison with total length  $l$  of the chromosome. The occurrence of each break results in a definite lowering of the probability of the next break.

Since the two chromatids involved in an interchange may be supposed to have identical elastic properties, we may, for the purpose of mathematical formulation, treat the problem as that of a single elastic thread along which breaks occur. After each break the ends are supposed to reunite again, resulting in the exclusion of a further break, in a region of length  $\epsilon$  round the location of the break and in a lowering of the probability for the next break.

The assumption about the effect of the occurrence of a break on the probability of occurrence of the next break may be formulated more precisely as follows: If  $\sigma$  is the breaking load and  $f_r(\sigma)$  the probability-density of  $\sigma$  after the  $r$ th break, then  $\int_0^S f_r(\sigma) d\sigma$  is the chance of the thread not exceeding  $S$  in strength, after the  $r$ th break. Evidently this chance diminishes with increasing  $r$ , in conformity with the assumption made here that each break lessens the probability of the next. Specifically, it is assumed that

$$\int_0^S f_{r+1}(\sigma) d\sigma = [(l - r\epsilon)/l] \int_0^S f_r(\sigma) d\sigma. \quad (1)$$

According to the assumptions made, each break eliminates a segment  $\epsilon$  of the length available for further breaks. These are equivalent to assuming that the chance of the thread not exceeding  $S$  in strength is reduced at each break in the ratio of the available effective length of the thread to the total length. The reduction-factor  $(l - r\epsilon)/l$  in (1) may also be rendered plausible by the following argument: if we assume this factor to be of the form  $g(\epsilon)$ , we may, on account of the smallness of  $\epsilon$ , write

$$g(\epsilon) = a + b\epsilon.$$

In the absence of interference, i.e. for  $\epsilon = 0$ , we must have  $g(0) = 1$ . Thus  $a = 1$  and, since  $g$  must be non-dimensional, we must put  $b = -k/l$ . This gives an expression of the above form. Since (1) is true for any  $S$ , it is equivalent to

$$f_{r+1}(\sigma) = \frac{l - r\epsilon}{l} f_r(\sigma). \quad (2)$$

We are now in a position to determine the probability of  $n$  breaks. If  $\sigma_1, \sigma_2, \dots, \sigma_n$  are the strengths of the thread at the 1st, 2nd,  $\dots$   $n$ th breaks, under the longitudinal

stress  $S$ , then the probability  $\phi_n$  of  $n$  breaks is following a procedure due to Daniels (1945):

$$\begin{aligned}\phi_n &= P \int_0^S f_1(\sigma_1) d\sigma_1 \int_{\sigma_1}^S f_2(\sigma_2) d\sigma_2 \dots \int_{\sigma_{n-1}}^S f_n(\sigma_n) d\sigma_n \\ &= P \frac{(l-\epsilon)(l-2\epsilon)\dots(l-n\epsilon)}{l^n} \int_0^S f_1(\sigma_1) d\sigma_1 \int_{\sigma_1}^S f_1(\sigma_2) d\sigma_2 \dots \int_{\sigma_{n-1}}^S f_1(\sigma_n) d\sigma_n \\ &= \frac{(l-\epsilon)(l-2\epsilon)\dots(l-n\epsilon)}{l^n} \frac{P}{L^n} [F(S) - F(0)]^n,\end{aligned}$$

where  $P$  is a factor to be determined presently and  $F(x) = \int_0^x f_1(x) dx$ .

If we set  $\lambda = F(S) - F(0)$ ,

$$\phi_n = \frac{(l-\epsilon)(l-2\epsilon)\dots(l-n\epsilon)}{l^n} P \cdot \frac{\lambda^n}{L^n} \quad (3)$$

To determine  $P$  the following condition is used.

$$\sum_0^\infty \phi_n = 1.$$

This gives

$$P = \left(1 + \frac{\lambda\epsilon}{l}\right)^{-[l/\epsilon]+1}$$

$$\text{so that, finally, } \phi_n = \frac{(l-\epsilon)(l-2\epsilon)\dots(l-n\epsilon)}{l^n} \left(1 + \frac{\lambda\epsilon}{l}\right)^{[l/\epsilon]+1} \frac{\lambda^n}{L^n}. \quad (4)$$

Actually  $n$  can only take the values  $0, 1, 2, \dots, N$ , where  $N = [l/\epsilon]$  is an integer such that  $N \leq l/\epsilon < N+1$ . The probability  $\phi_n$  is then conditioned by  $\sum_0^N \phi_n = 1$ . Thus

$$P \sum_0^N \frac{(l-\epsilon)(l-2\epsilon)\dots(l-n\epsilon)}{l^n} \frac{\lambda^n}{L^n} = 1,$$

$$\text{i.e. } P \sum_0^N \frac{\left(\frac{l}{\epsilon}-1\right)\left(\frac{l}{\epsilon}-2\right)\dots\left(\frac{l}{\epsilon}-n\right)}{L^n} \left(\frac{\epsilon\lambda}{l}\right)^n = 1.$$

Let  $l/\epsilon = N + \alpha$ ,  $0 \leq \alpha < 1$ . If  $\epsilon \ll l$ ,  $N$  will be large, and  $\alpha$  small. Then the above condition becomes

$$P \sum_0^N \frac{(N+\alpha-1)(N+\alpha-2)\dots(N+\alpha-n)}{L^n} \left(\frac{\epsilon\lambda}{l}\right)^n = 1.$$

Since  $N$  is large and  $\alpha$  small, we may neglect  $\alpha$  in each factor in the numerator. This gives

$$P \sum_0^N \frac{(N-1)(N-2)\dots(N-n)}{L^n} \left(\frac{\epsilon\lambda}{l}\right)^n = 1,$$

or

$$P \sum_0^{N-1} \frac{(N-1)(N-2)\dots(N-n)}{L^n} \left(\frac{\epsilon\lambda}{l}\right)^n = 1.$$

Thus, finally, we have

$$P\left(1 + \frac{\epsilon\lambda}{l}\right)^{N-1} = 1 \quad \text{or} \quad P = (1 + \epsilon\lambda/l)^{-N+1}$$

and the expression for the probability  $\phi_n$  becomes

$$\phi_n = \frac{(l-\epsilon)(l-2\epsilon)\dots(l-n\epsilon)}{l^n} \left(1 + \frac{\lambda\epsilon}{l}\right)^{-l/\epsilon+1} \frac{\lambda^n}{L^n} \quad (5)$$

For  $\epsilon \ll l$ , this practically coincides with (4). The corresponding generating function  $\psi(t)$  is given by

$$\psi(t) = \sum_0^N \phi_n t^n = \left(1 + \frac{\epsilon\lambda}{l}\right)^{-N+1} \left(1 + \frac{\epsilon\lambda t}{l}\right)^{N-1}. \quad (6)$$

From (5) we see that, as  $\epsilon \rightarrow 0$ ,

$$\phi_n \rightarrow e^{-\lambda} \frac{\lambda^n}{L^n} \quad (7)$$

which is the Poisson distribution, so that in the absence of interference, the distribution reduces to the Poisson type. It will also be noticed that the same conclusion would follow from (5) if, keeping  $\epsilon$  fixed, we let  $l \rightarrow \infty$ . In other words there should be no interference in an infinitely long thread. This is in conformity with our general expectations, since, in an infinitely long thread, a break can *always* occur at a sufficient distance from previous ones, so as to avoid the effect of interference of these. The fact that independence from interference is attained for sufficiently long chromosomes is a well established experimental result Muller (1916).

We proceed to calculate the mean and standard deviation for the distribution (5). The mean  $m$  is given by

$$\begin{aligned} m &= \sum_0^N n\phi_n = \left[ \frac{d}{dt} \psi(t) \right]_{t=1} \\ &= \left(1 + \frac{\epsilon\lambda}{l}\right)^{-N+1} (N-1) \left(1 + \frac{\epsilon\lambda}{l}\right)^{N-2} \frac{\epsilon\lambda}{l} \\ &= \frac{(N-1)(\epsilon\lambda/l)}{1 + (\epsilon\lambda/l)}. \end{aligned}$$

Writing  $N = l/\epsilon$ , approximately, we have

$$m = \frac{\lambda \left(1 - \frac{\epsilon}{l}\right)}{1 + \frac{\epsilon\lambda}{l}}. \quad (8)$$

Similarly the standard deviation  $s^2$  is given by

$$\begin{aligned} s^2 &= \sum_0^N (m-n)^2 \phi_n \\ &= m^2 \sum_0^N \phi_n - 2m \sum_0^N n\phi_n + \sum_0^N n^2 \phi_n \\ &= -m^2 + \sum_0^N n^2 \phi_n. \end{aligned}$$

Now 
$$\Sigma n^2 \phi_n = \left[ \frac{d}{dt} \left\{ t \frac{d\psi}{dt} \right\} \right]_{t=1}$$

$$= \frac{\frac{\epsilon\lambda}{l} \left( \frac{l}{\epsilon} - 1 \right)}{\left( 1 + \frac{\epsilon\lambda}{l} \right)^2} \left[ 1 + \lambda \left( 1 - \frac{\epsilon}{l} \right) \right] \quad \text{with } N \sim \frac{l}{\epsilon},$$

and hence

$$s^2 = \frac{\lambda \left( 1 - \frac{\epsilon}{l} \right)}{\left( 1 + \frac{\epsilon\lambda}{l} \right)^2}. \quad (9)$$

For  $\epsilon \rightarrow 0$ ,  $m = s^2 = \lambda$ , so that the variance and the mean coincide, which is a characteristic of the Poisson distribution. The equations (8) and (9) put in evidence the effect of interference on the mean and the variance, which are connected by the relation

$$\bullet \quad m^2 = \lambda \left( 1 - \frac{\epsilon}{l} \right) s^2. \quad (10)$$

The relation (8) also gives the chromosome length mean chiasma frequency relationship. The theoretically derived curve (Mather 1938), however, shows a departure from the experimental curve, the reason for which is under examination. This contribution is offered as a first attempt at a theoretical formulation of a difficult biological problem. Grateful thanks are due to Mr U. R. Thiruvengatchar for his valuable assistance in working out this problem.

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# The route of antibodies passing from the maternal to the foetal circulation in rabbits

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It has long been known that maternal circulating antibodies pass into the foetal blood in rabbits during the latter half of pregnancy. The allanto-chorionic placenta has been assumed to be the site of this transference, the number of tissues separating the two blood streams being reduced to a minimum in rabbits at these stages. It was shown in a recent paper that, at a stage before the establishment of the embryonic circulation, maternal circulating antibodies pass the bilaminar omphalopleur into the yolk-sac cavity. It is shown in this paper that in 24-day embryos antibodies pass from the maternal circulation by way of the uterine lumen and the yolk-sac splanchnopleur into the foetal vitelline circulation, and do not pass by way of the allanto-chorionic placenta. The method employed involved injection of immune rabbit serum either into the uterine lumen or the maternal blood and interruption of the foetal vitelline circulation of some of the embryos by ligaturing the yolk-sac stalk.

## INTRODUCTION

It has been shown previously (Brambell & Hemmings, with McCarthy & Kekwick, 1949; Brambell, Hemmings & Rowlands 1948) that plasma proteins, including specific antibodies (agglutinins), pass freely from the maternal circulation through the bilaminar omphalopleur into the yolk-sac cavity of 7- and 8-day rabbit embryos. It is well known that young rabbits acquire a passive immunity from the mother mainly before birth, with some possible augmentation later via the colostrum. It has been shown that in actively immunized rabbits the agglutinin titre in the embryonic blood continues to rise from the 22nd day to full term (Rodolfo 1934) and the passage of agglutinins passively acquired by the mother after the 15th day has been observed (Brambell *et al.* 1948).

These results reopened the problem as to whether the antibodies pass into the embryonic circulation in rabbits by way of the allanto-chorionic placenta, as has been generally assumed heretofore, or by way of the only other available route, the yolk-sac splanchnopleur, or both. The presence of antibodies in the yolk-sac cavity, before the allanto-chorionic placenta is established, clearly suggested the latter as possible. The fact that antibodies pass rapidly through the gut wall from the first feed of colostrum into the circulation in new-born ruminants supports this suggestion, because the yolk-sac splanchnopleur is morphologically an extra-embryonic extension of the mid-gut wall. In the rabbit the allanto-chorionic placenta is fully established by the 15th day, by which time the bilaminar omphalopleur has disrupted, exposing the entoderm of the everted splanchnic wall to the uterine lumen. The original uterine epithelium of the antimesometrial or

obplacental region, which was invaded by the omphaloidean trophoblast, has disintegrated, with the omphalopleur, and a new epithelium has been regenerated, with which the exposed yolk-sac entoderm is neither fused nor in contact. Thus the solution of the problem of transmission by this route requires answers to two questions. First, do antibodies pass from the uterine lumen through the yolk-sac splanchnopleur into the embryonic circulation? Secondly, if so, do they pass from the maternal circulation into the uterine lumen during pregnancy? The experiments described herein were designed to provide answers to these questions.

The first experiment consisted of the injection of *Brucella abortus* immune serum directly into the lumen of one of the two uterine horns, the other horn serving as a control. The second experiment was a repetition of the first after interrupting the vitelline circulation of some, but not all, embryos on the experimental side. The third experiment consisted of the intravenous injection of immune serum into the mother after interruption of the vitelline circulation of some of the embryos. It was appreciated that this last experiment alone, if successful, was capable of providing all the information required, but the possible technical hazards involved justified the cautious approach by way of the simpler experiments.

#### EXPERIMENTAL TECHNIQUE

The majority of the animals used were Dutch rabbits of the Agricultural Research Council's Compton strain. A few, of various breeds, were purchased from the trade.

Many, but not all, of the animals were blood tested prior to use and were invariably found to contain no specific agglutinins for *Br. abortus*. In every case, except no. 208, the negative titre in some of the embryos provides adequate evidence of the absence of agglutinins prior to injection; each animal therefore was self controlled.

The operations were performed at 23 to 26 days post-coitum, 24 days being found to be the optimum since this was the earliest stage at which sufficient serum could be collected from each embryo for testing. Anaesthesia was effected by intravenous injection of up to 1 gr./kg. live weight of veterinary Nembutal followed by ether. A dose of 0.01 gr./kg. live weight of atropine, administered intravenously 30 min. before operation to reduce mucous secretion in the respiratory tract, diminished the risk of respiratory failure under prolonged anaesthesia and was employed in all the experiments involving intra-uterine injection. It was omitted in the experiments involving intravenous injection of serum on account of the probable reduction of uterine secretion. A dose of 0.25 ml. of *l. adrenalin* (B.P.) was usual whenever breathing became unduly laboured. Amphetamine sulphate was administered during or after the operation in doses of 0.1 gr. to 0.25 gr. subcutaneously or intravenously when required as a cardiac stimulant. The operations were performed with aseptic precautions. An incision was made in the mid-ventral line and the gravid uterus partly or wholly exteriorized and wrapped in warm gauze dressings wetted in saline. In conceptuses at this stage the umbilical vessels divide into the allantoic and vitelline vessels within 1 cm. of the umbilicus,



the allantoic vessels branching and running round the side walls of the thin allantoic vesicle to the mesometrial allanto-chorionic placenta (figure 1). The vitelline vessels run laterally between the conjoined amnion and allantois and, from the point where these membranes diverge, they extend freely across the cavity of the exocoel as the long yolk-sac stalk which joins the yolk-sac splanchnopleur at a point on the circumference of the membranes two-thirds of the way from the mesometrial to the antimesometrial pole. This part of the yolk-sac stalk which is free in the exocoel runs round the embryo just behind the shoulder. The vitelline vessels radiate over the surface of the area vasculosa from the junction of the yolk-sac stalk. The uterine wall is sufficiently stretched and transparent to permit of seeing clearly through it both the yolk-sac stalk and the point, marked by the radiating vessels, where it joins the yolk-sac splanchnopleur. This point lies approximately 0.5 cm. to the antimesometrial side of the lateral uterine vein, which is conspicuous. By carefully lifting a small fold of uterine wall immediately over this point, a small radial incision 2 to 3 mm. long could be made without injuring the embryonic membranes beneath. These immediately bulged through the hole and, if this was well placed, the exposed portion included the place where the yolk-sac stalk joined the splanchnopleur. Seizing this with fine curved forceps with rubber-covered tips it was easy to ligature the yolk-sac stalk a few mm. from its end without rupturing the yolk-sac splanchnopleur. Two silk ligatures, placed a couple of mm. apart were employed in each case. The silk ends were cut off short, the edges of the uterine incision lifted with forceps and the membranes gently returned to the uterine lumen. The incision was sutured with two or three stitches of silk, the sutured incision then pinched up in forceps and a silk ligature secured around the neck of the small fold, thus completely isolating the incision. The suture facilitated placing this ligature and prevented it slipping off subsequently, and the ligature prevented bleeding from the incision. Little difficulty was experienced in performing this operation without loss of embryonic fluid and with negligible uterine haemorrhage, provided care was taken to avoid interrupting the lateral uterine vein. Two, three or four embryos in each experimental animal were treated in this way and the mortality in the 24 to 48 hr. over which the experiment was continued was no greater in the operated, than in the unoperated, embryos on the experimental side or in those in the unopened control uterus. Noer & Mossman (1947) have found in the rat that tying the vitelline vessels did not result in death of the foetuses within 3 hr., as did tying the allantoic vessels, but did result in death within 4 days. Our experiments were not continued over so long a period but the vitality of the embryos at the conclusion appeared unimpaired. Death of embryos, both experimental and control, as a result of the operation was attributable either to the effects of anaesthesia or to too prolonged vascular stasis in the uterus due to pressure on the main uterine vessels resulting from exteriorization.

Serum was injected into the uterine lumen between conceptuses, through a fold of the uterine wall pinched up between the fingers. It was necessary to exercise great care to avoid puncture of the membranes, injection into the uterine mucosa, or leakage of serum from the puncture. The uterine wall around the puncture

point was ligatured immediately after withdrawal of the needle. For this purpose rabbit anti-serum to *Br. abortus* with an agglutinin titre of  $1:280$  to  $1:2560$  was employed.

Intravenous injections of serum were administered through an ear vein after the laparotomy was completed.

The animal was killed by intravenous injection of magnesium chloride to secure inactivation of the uterine musculature, a sample of maternal blood being drawn from an ear vein or from the heart at the same time. The uterus was immediately exposed and samples of embryonic fluids, heart blood and stomach contents were drawn from each conceptus in turn, clean pipettes being used to draw each sample. Despite care one could not be sure of obtaining exocoel, amniotic and allantoic fluids unmixed. Even when the cavity from which the fluid was drawn was identified with certainty the possibility of rupture of one of the bounding membranes, permitting contamination, could not be entirely excluded. The agglutinin titre of each of the samples was determined by the standardized technique described previously (Brambell *et al.* 1948).

## RESULTS

### *Intra-uterine injection of immune serum*

Injection of rabbit serum of high anti-*Brucella* agglutinin titre into the lumen of one uterus was performed on two animals 24 days pregnant to determine if the antibodies would pass into the embryonic circulation. Both animals yielded decisive positive results, the sera of all the embryos in the experimental uteri having high titres, and the sera of none of the embryos in the control uteri having detectable titres. It was concluded that the agglutinin was passing directly into the foetal circulation from the uterine lumen and not indirectly by way of the maternal circulation.

The omphaloidean vessels of some of the embryos in the experimental uterus were ligatured in the remainder of the experiments on intra-uterine injection of immune serum. The results concerning the serum of both these series of experiments are summarized in table 1 where embryos with the vessels ligatured are indicated by a cartouche around the symbols for the titre of the serum. It can be seen by inspection of this table that the sera of all the untouched embryos in the experimental uteri had a highly significant positive agglutinin titre of not less than  $+++ \frac{1}{40}$ , whereas the sera of all the embryos in the control uteri were negative at the lowest dilution tested, which was  $\frac{1}{10}$  in all except one where the amount of serum recovered was so small as to necessitate an initial dilution of  $\frac{1}{20}$ . The evidence for the direct passage of agglutinins from the uterine lumen into the embryonic circulation is conclusive. The sera of the majority of the embryos in which the vitelline vessels were tied were negative at a dilution of  $\frac{1}{10}$ , demonstrating that complete interruption of the circulation of the area vasculosa can preclude the entry of agglutinins into the embryonic blood. A significant agglutinin titre was found in the sera of some of the embryos in which the vitelline vessels were tied. This was puzzling at first, since the ligatures never became displaced, but

examination of the area vasculosa of each embryo at autopsy revealed that although in the majority of instances ligation resulted in interruption of the vitelline circulation, with stagnation and darkening of the blood in all the vessels, in a minority some or all of the vessels distal to the ligatures remained apparently normal and evidently contained circulating blood, although the ligatures were still in place. All embryos in which active circulation in the area vasculosa was noted at autopsy, as well as a few in which it was not, had a positive agglutinin titre in the serum. It is not easy to understand how, in some cases, the vitelline circulation is maintained or restored after ligaturing.

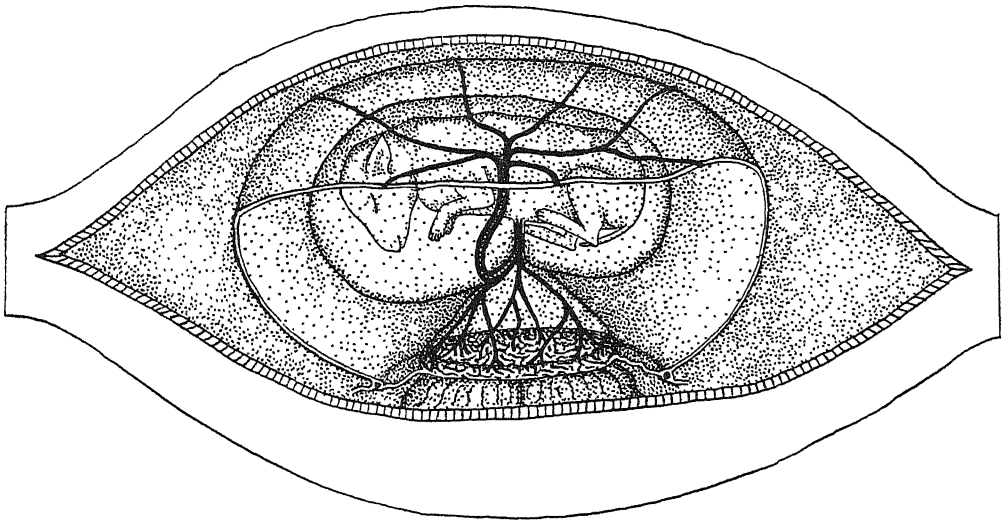


FIGURE 1. Diagram of a 24-day embryo rabbit in its membranes within the uterus. The uterus has been opened laterally with the mesometrium on the lower side, exposing the membranes in the uterine lumen. The lower half of the splanchnopleur on the near side has been removed, opening the exocoel and giving a clear view of the placenta and allantois below and of the yolk-sac stalk with the vitelline vessels bending upwards in the exocoel. The junction of the yolk-sac stalk with the intact upper half of the yolk-sac splanchnopleur can be seen close behind the shoulder of the embryo. The vitelline vessels radiate from this point over the surface of the area vasculosa. The sinus terminalis can be seen cut across on each side of the placenta, marked by the tag which is all that remains of the bilaminar omphalopleur. Between the sinus terminalis and the placenta the exocoel is bounded by chorion.

Anastomoses are known to occur between the vitelline and allanto-chorionic vessels around the periphery of the area vasculosa, where it adjoins the margin of the discoid allanto-chorionic placenta (Duval 1892). We also have observed such anastomoses but have been unable to determine if they are invariably present. The effectiveness of ligaturing the vitelline vessels as a means of stopping the circulation in the area vasculosa in any individual embryo may depend on whether these anastomoses are present and of sufficient size and suitably disposed to maintain the circulation of a part or the whole of the area vasculosa. We observed that ligation resulted in darkening of the vessels in the centre of the area vasculosa but not at the periphery in some instances. The fact that the agglutinin titres in the

TABLE 1. INTRA-UTERINE INJECTION OF IMMUNE SERUM

ref. no.	age at operation (days)	duration of experiment (hr.)	injected serum		maternal serum at killing (titre)	titre of embryonic serum	
			dose (ml.)	titre		experimental uterus	control uterus
106	24	27	6	++ + $\frac{1}{2560}$	+	$\frac{1}{20}$ + ++ $\frac{1}{160}$	— $\frac{1}{16}$ . . . .
209	24	23	7	++ + $\frac{1}{2560}$	++ + $\frac{1}{160}$ + $\frac{1}{160}$	+	— $\frac{1}{16}$ — $\frac{1}{16}$ — $\frac{1}{16}$ .
207	23	43	6	++ + $\frac{1}{2560}$	— $\frac{1}{16}$	dead	dead — $\frac{1}{16}$ . . . .
206	24	47	6	++ + $\frac{1}{2560}$	— $\frac{1}{16}$ + ++ $\frac{1}{320}$	++ + $\frac{1}{160}$ + + $\frac{1}{80}$	— $\frac{1}{16}$ — $\frac{1}{16}$ — $\frac{1}{16}$ .
203	24	44	6	++ + $\frac{1}{1280}$	— $\frac{1}{16}$ + ++ $\frac{1}{160}$	+	— $\frac{1}{16}$ — $\frac{1}{16}$ . . . .
213	26	22	6	++ + $\frac{1}{1280}$	— $\frac{1}{16}$ + $\frac{1}{160}$	++ + $\frac{1}{160}$ + $\frac{1}{16}$	— $\frac{1}{16}$ — $\frac{1}{16}$ . . . .
184	24	24	6	++ + $\frac{1}{2560}$	— $\frac{1}{16}$ + ++ $\frac{1}{40}$	++ + $\frac{1}{40}$	— $\frac{1}{16}$ — $\frac{1}{16}$ . . . .
143	24	25	5	++ + $\frac{1}{1280}$	— $\frac{1}{16}$ + ++ $\frac{1}{40}$	— $\frac{1}{16}$	— $\frac{1}{16}$ — $\frac{1}{16}$ . . . .
204	24	20	7	++ + $\frac{1}{320}$	— $\frac{1}{16}$ dead	dead — $\frac{1}{16}$	dead dead ? — $\frac{1}{16}$ — $\frac{1}{16}$
120	24	20	7	++ + $\frac{1}{2560}$	— $\frac{1}{16}$ dead	dead	— $\frac{1}{16}$ dead . . . .
121	24	20	6	++ + $\frac{1}{2560}$	— $\frac{1}{16}$ + ++ $\frac{1}{160}$	+	— $\frac{1}{16}$ dead . . . .
220	24	20	7	++ + $\frac{1}{1280}$	— $\frac{1}{16}$ + ++ $\frac{1}{80}$	+	— $\frac{1}{16}$ removed — $\frac{1}{16}$ dead .
155	24	24	7	++ + $\frac{1}{1280}$	— $\frac{1}{16}$ + $\frac{1}{80}$ + $\frac{1}{160}$	dead + + + $\frac{1}{80}$	— $\frac{1}{16}$ — $\frac{1}{16}$ . . . .

TABLE 2. INTRAVENOUS INJECTION OF IMMUNE SERUM

ref. no.	age at operation (days)	duration of experiment (hr.)	injected serum		maternal serum at killing (titre)	titre of embryonic serum	
			dose (ml.)	titre		right uterus	left uterus
135	24	23	45	++ + $\frac{1}{2560}$	++ + $\frac{1}{320}$	— $\frac{1}{16}$ + ++ $\frac{1}{20}$ + $\frac{1}{40}$	++ + $\frac{1}{40}$ + ++ $\frac{1}{40}$ + ++ $\frac{1}{40}$ .
151	24	22	40	++ + $\frac{1}{1280}$	++ + $\frac{1}{160}$	++ + $\frac{1}{20}$ + ++ $\frac{1}{40}$	++ + $\frac{1}{16}$ + ++ $\frac{1}{40}$ .
229	24	21	40	++ + $\frac{1}{1280}$	++ + $\frac{1}{320}$	— $\frac{1}{16}$ + $\frac{1}{40}$	aborted . . . .
208	24	28	40	++ + $\frac{1}{1280}$	++ + $\frac{1}{320}$	++ + $\frac{1}{16}$ + $\frac{1}{40}$	++ + $\frac{1}{40}$ . . . .
523	24	24	36	++ + $\frac{1}{2560}$	++ + $\frac{1}{160}$	++ + $\frac{1}{40}$ + ++ $\frac{1}{40}$	— $\frac{1}{16}$ + ++ $\frac{1}{40}$ + ++ $\frac{1}{40}$ .
290	24	23	45	++ + $\frac{1}{2560}$	++ + $\frac{1}{160}$	++ + $\frac{1}{40}$ + ++ $\frac{1}{80}$	++ + $\frac{1}{16}$ + ++ $\frac{1}{80}$ + ++ $\frac{1}{40}$ .
200	24	24	43	++ + $\frac{1}{2560}$	++ + $\frac{1}{320}$	— $\frac{1}{16}$ + ++ $\frac{1}{80}$ + $\frac{1}{160}$	— $\frac{1}{16}$ + ++ $\frac{1}{80}$ . . . .

sera of those embryos which were positive, after tying the vitelline vessels, was as a rule significantly lower than in the sera of the normal embryos from the same horn shows that the circulation was only partially interrupted. Since the ligature is adjusted without penetrating the membranes the close approximation of vitelline vessels distal and proximal to it cannot be avoided, as can be appreciated by reference to figure 2. The endothelial walls of the vessels are very thin and are separated from the exocoel only by an equally thin mesothelium, hence it is possible that anastomoses could be formed at this point, re-establishing the normal vitelline circulation and by-passing the ligature altogether.

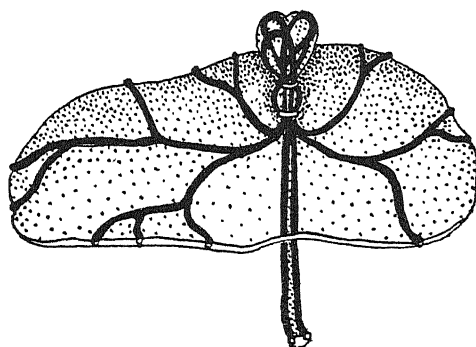


FIGURE 2. Diagram of a part of the yolk-sac stalk and of the splanchnopleur of the area vasculosa surrounding the point where they join. Two ligatures have been fixed to show the way in which the vessels are tied without perforating the membranes.

The titre in the embryonic serum does appear to be related to the titre of the serum injected, since titres of  $\frac{1}{160}$  to  $\frac{1}{320}$  were obtained after injection of  $\frac{1}{2560}$  serum, whereas titres of  $\frac{1}{40}$  to  $\frac{1}{160}$  resulted from injections of  $\frac{1}{1280}$  serum. No such obvious relations appear to exist, under the conditions of the experiment, between the titre of the embryonic serum, and the quantity of serum injected, number of embryos exposed to it or time of exposure, suggesting that the dose of serum may have been in excess of the capacity of the embryos to absorb it.

It is significant that agglutinins were detected in the maternal serum of only two of the thirteen experimental animals. In one of these (no. 209) some leakage of serum from the uterine puncture on to the peritoneal surface was detected at the time of injection. Since the possibility of some such leakage or of injection of part of the dose into the tissues of the uterine mucosa instead of into the lumen cannot be entirely precluded even by exercise of the most meticulous care, two out of thirteen positives cannot be regarded as satisfactory evidence of the passage of antibodies from the uterine lumen into the maternal circulation. Since dilution of 1 ml. of serum with a titre of  $\frac{1}{2560}$  in the maternal blood volume of *ca.* 120 ml. should give a titre of *ca.*  $\frac{1}{20}$  to  $\frac{1}{40}$ , the results indicate, on the contrary, that little if any absorption of antibodies from the uterine lumen into the maternal circulation occurs about the 24th day of pregnancy in rabbits.

*Intravenous injection of immune serum*

The results of the third series of experiments in which immune serum was injected intravenously into pregnant rabbits immediately after the vitelline vessels of some of the contained embryos had been ligatured are summarized in table 2. It is apparent that all the untied embryos developed a titre of  $\frac{1}{20}$  to  $\frac{1}{80}$ , showing clearly that agglutinins pass readily from the maternal into the embryonic blood. Hence the agglutinins in the maternal circulation must either pass through the allanto-chorionic placenta or be exuded or secreted from the uterine wall into the uterine lumen, thence passing into the embryonic blood in the area vasculosa. Since no agglutinins were detectable at a dilution of  $\frac{1}{10}$  in the serum of nine out of the eighteen embryos in which the vitelline vessels were ligatured, it is evident that in these the allanto-chorionic placenta was an effective barrier. The remainder of the embryos in which the vitelline vessels were ligatured had agglutinins in the serum at a titre which in most instances was significantly lower than in the unligatured embryos of the same litter. It is, of course, theoretically possible that in these the antibodies passed through the allanto-chorionic placenta but there is no reason to suppose that incomplete interruption of the vitelline circulation did not account for the leakage, as in the experiments on injection of immune serum into the uterine lumen. Indeed, it was observed at autopsy that the circulation was maintained in some, but not in all, of these embryos with ligatured vitelline vessels in which agglutinins had passed from the maternal into the foetal circulations.

It must be concluded from these results that antibodies can pass from the maternal circulation into the uterine lumen on the 25th day of gestation in rabbits. That this passage is not confined to exudation from the surgical lesions in the uterine wall is shown by the fact that the titres in the sera of the embryos in the operated uteri were not significantly higher than those of the embryos in the unopened uteri of rabbits nos. 135 and 151. One animal, no. 208, was pregnant in one uterus only and provided an opportunity, unfortunately unique in this series of experiments, of demonstrating directly the passage of antibodies into the uterine lumen. The non-gravid uterus in this animal was ligatured above the cervix, care being taken to exclude the main uterine vessels from the ligature, at the same time as the vitelline vessels of two of the three embryos in the gravid uterus were ligatured. Fluid collected in the lumen of this non-gravid uterus and was aspirated at autopsy. This uterine fluid had an agglutinin titre of + + + at  $\frac{1}{40}$ , compared to a titre in the blood serum of + + + at  $\frac{1}{320}$  at autopsy and + + + at  $\frac{1}{640}$  when at a maximum shortly after injection of the immune serum.

*The passage of antibodies into the extra-embryonic fluids and stomach contents of embryos*

So far as was possible, samples of the exocoelomic, amniotic and allantoic fluids and of the stomach contents of embryos were collected at autopsy and tested for agglutinins in each series of experiments. Agglutinins were invariably absent from all the samples of these fluids from the embryos in the control uterine horns of the series involving intra-uterine injection of immune serum. Agglutinins were absent

from the samples of allantoic fluid in all embryos, experimental or control, as might be expected. Agglutinins were frequently present in the amniotic fluid. The titre did not appear to be correlated with that of the foetal serum, as can be seen from table 3 summarizing the data for 78 embryos. The exocoelomic fluid in none of the 17 embryos for which data are available had a lower titre than that of the corresponding amnion although in a few instances it was slightly higher. These results suggest that agglutinins pass from the uterine lumen directly through the splanchnopleur into the exocoelomic fluid and thence through the amnion into the amniotic fluid, rather than via the foetal circulation. The reason that agglutinins were found in the amniotic fluid in 40 of the 78 embryos and not in the remaining 38 is not apparent. Agglutinins were detected in the amniotic fluid of 20 out of 34 embryos from the series of intra-uterine injections and in 20 out of 44 embryos from the series of intravenous injections; they were present in the amniotic fluid of 15 out of 30 embryos in which the vitelline vessels were ligatured, and of 25 out of 48 in which the vessels were not ligatured. It is possible that we are dealing here with individual idiosyncrasies of the embryos analogous to those determining the secretion or non-secretion of antibodies in the saliva and other body-fluids of adults of various species.

TABLE 3

titre of serum	titre of amniotic fluid					total
	$> \frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	
$\frac{1}{320}$	1	.	.	.	.	1
$\frac{1}{160}$	4	5	2	.	.	11
$\frac{1}{80}$	3	7	2	.	.	12
$\frac{1}{40}$	15	9	2	.	.	26
$\frac{1}{20}$	2	1	1	.	.	4
$\frac{1}{10}$	2	.	.	2	.	4
$> \frac{1}{10}$	11	3	3	2	1	20
total	38	25	10	4	1	78

Agglutinins were detected in the stomach contents of 43 out of 77 embryos. Their occurrence and titre in the stomach contents and in the amniotic fluid appear to be correlated, as is shown by table 4. In one instance, not included in the table, a very high titre of + + + at  $\frac{1}{1280}$  in the stomach contents was due almost certainly to direct injection of immune serum into the embryonic membranes, suspected at the time of injection. It can be seen that in 6 other embryos in which agglutinins were not detected in the amniotic fluid agglutinins were present in the stomach contents and in a further 13 embryos the titre of the stomach contents was higher than that of the amniotic fluid. It would appear that there is a tendency for the agglutinins to be concentrated in the stomach, either by differential absorption of water or by adsorption on mucus, probably from amniotic fluid which is swallowed. The occurrence and titre of agglutinins in the stomach contents and serum do not appear to be correlated, as can be seen from the data in table 5. Since the foetal circulation would appear to be the only other feasible route by which the agglutinins could reach the stomach contents this conclusion supports the assumption that they are derived from the amniotic fluid.

The somewhat unexpected results obtained with the stomach contents raised the question as to whether this might be the result of some non-specific reaction due to their mucin content. In order to clear up this point, as far as possible, a number of samples of stomach contents were set up in duplicate, using, in addition to the standard *Brucella* antigen, a standard antigen prepared against *Salmonella pullorum*, an easily agglutinable organism causing a specific enteric disease of young chicks. No agglutination of this organism took place in any dilution. This result was accepted as reasonable proof of the specificity of the original reactions.

TABLE 4

titre of amniotic fluid	titre of stomach contents						total
	$> \frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	
$\frac{1}{40}$	.	.	.	3	2	.	5
$\frac{1}{20}$	1	1	5	2	.	.	9
$\frac{1}{10}$	4	12	8	1	.	.	25
$> \frac{1}{10}$	28	2	2	1	.	1	34
total	33	15	15	7	2	1	73

TABLE 5

titre of serum	titre of stomach contents						total
	$> \frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	
$\frac{1}{320}$	.	.	1	.	.	.	1
$\frac{1}{160}$	3	2	3	2	.	.	10
$\frac{1}{80}$	3	6	2	1	.	.	12
$\frac{1}{40}$	19	4	4	.	.	2	29
$\frac{1}{20}$	2	1	1	.	.	.	4
$\frac{1}{10}$	1	.	.	2	.	.	3
$> \frac{1}{10}$	6	2	4	2	3	1	18
total	34	15	15	7	3	3	77

### CONCLUSIONS

The experiments on intra-uterine injection of immune serum demonstrate that agglutinins present in the uterine lumen pass rapidly into the foetal blood in the area vasculosa of the yolk-sac splanchnopleur of 23 to 26 day old rabbit embryos. This occurs without exception in all the embryos in which the vitelline circulation is intact and which are exposed to the immune serum. This capacity of the yolk-sac splanchnopleur of rabbit embryos to admit antibodies into the circulation is shared with the gut of the new-born young of many mammals through which antibodies from the colostrum pass rapidly into the circulation. This is not surprising since, morphologically, the yolk-sac splanchnopleur is an extra-embryonic extension of the mid-gut wall, and, in fact, this consideration led us to predict the possibility (Brambell *et al.* 1948). It is possible that the transitory permeability to antibodies of the gut of the new-born should be regarded as a neonatal extension of what may be a characteristic property of the antenatal splanchnopleur, both intra- and extra-embryonic.



Two considerations indicate that the agglutinins pass from the uterine lumen directly into the blood in the vessels, and not indirectly by way of the other embryonic fluids. First, the occurrence of agglutinins in the exocoelomic and amniotic fluids of embryos exposed to immune serum in the uterine lumen is very erratic, only *ca.* 50 % showing a positive titre, in sharp contrast to the regularity of their occurrence in the blood. Secondly, successful experimental interruption of the vitelline circulation precludes their entry. Direct entry from the uterine lumen into the vitelline vessels involves passage, first through the cellular layer of yolk-sac entoderm, which is at this stage in the form of a regular cubical or low columnar epithelium that appears to be uninterrupted throughout the extent of the area vasculosa, then through the thin layer of splanchnic mesenchyme surrounding the vessels, and finally through their endothelial walls. The vessels themselves, with their covering of mesenchyme and entoderm, project into the uterine lumen, forming pronounced ridges on the exposed surface of the yolk-sac, thus presenting the maximum surface area.

The experiments on intravenous injection of immune serum demonstrate that agglutinins in the maternal circulation of 25-day pregnant rabbits appear in the uterine lumen. The uterine epithelium of the extra-placental region of the mucosa is intact at this stage of gestation. It has been regenerated about the 10th day, following its invasion and destruction at the time of implantation by the trophoblast of the bilaminar omphalopleur. Hence the agglutinins must traverse this layer. Little evidence was obtained as to whether they are actively secreted by the cells of the uterine glands or epithelium or whether they appear in a vascular transudate. The occurrence of agglutinins in the fluid recovered from the single non-gravid uterus encountered shows that the presence of placentae in the uterus is not a necessary condition for their appearance. The titre in this fluid, + + + at  $\frac{1}{40}$ , was only one-eighth of that in the maternal serum, which was + + + at  $\frac{1}{320}$ , a very interesting observation on which it would be rash to build until more data are available. It is also somewhat surprising, in relation to the problem of the permeability of the uterine mucosa to agglutinins, that the experiments on intra-uterine injection of immune serum did not provide clearer evidence of their passage from the lumen into the maternal circulation. It must be remembered, however, that the resulting dilution in a total blood volume of *ca.* 120 ml. would require the passage of the agglutinin content of approximately 0.5 ml. of immune serum with a titre of  $\frac{1}{2560}$  to be detectable, although this is a comparatively small amount in relation to the total internal surface area of a gravid uterus exposed to the serum.

The prevention of passage of agglutinins from the maternal circulation into the foetal blood by successful experimental interruption of the vitelline circulation shows that agglutinins do not pass through the allanto-chorionic placenta of rabbits on the 25th day of gestation in detectable amounts. This is, perhaps, the most important and surprising result obtained, for the placenta of the rabbit at this stage is, according to Mossman (1926), haemo-endothelial; the most advanced type known. It has been generally supposed, not unreasonably, that the functional efficiency of the placenta is related to the degree of reduction of tissue layers intervening between the maternal and foetal circulation, and this reaches a climax

in haemo-endothelial placentae. Hence the antenatal passage of antibodies in rabbit and man has been attributed to their respective haemo-endothelial and haemo-chorial placentae. Yet despite the possession of what is rightly regarded as the most specialized type of allanto-chorionic placenta known, our results show that maternal antibodies reach the rabbit embryo exclusively by way of the yolk-sac splanchnopleur. Obviously the route of antibodies into the human embryo requires reinvestigation but it cannot be the same as in the rabbit, for the yolk-sac is small and is isolated by the exocoel from the chorion, which remains intact throughout development. At least it is clear that the similarity of rabbit and man in the intra-uterine derivation of passive immunity from the mother is not explained by the similarity in placental architecture.

The occurrence of agglutinins in the exocoelomic and amniotic fluids and in the stomach contents of some of the embryos shows that they can pass through both the splanchnopleur and the amnion, their absence in others suggests that one or both of these membranes may exclude them. The fact that embryos exposed to high-titre serum invariably admit agglutinins to the circulation but only allow them to pass through the thin bounding membranes into the exocoel and amnion in 50 % of the individuals is remarkable, and recalls to mind the individual variation in adults in the capacity to secrete immune bodies in the saliva and other body fluids. The apparent capacity of the embryonic stomach to concentrate agglutinins from the amniotic fluid is an intriguing associated phenomenon deserving further investigation.

Finally it is worth emphasizing that the technique of intra-uterine injection in pregnant rabbits provides a means of introducing  $\gamma$ -globulins into the embryos without introducing them into the maternal circulation. If this holds for other proteins, the method may provide great opportunities of exposing the embryos to protein hormones, anti-rabbit-protein sera, etc., that could not be introduced by way of the maternal circulation.

#### SUMMARY

The problem of whether antibodies pass into the embryonic circulation by way of the allanto-chorionic placenta or by way of the yolk-sac splanchnopleur or by both routes was investigated experimentally by intra-uterine and by intravenous injection of immune rabbit serum into normal 24-day pregnant rabbits. A surgical technique for the interruption of the circulation of the area vasculosa by ligaturing the vitelline vessels without perforating the embryonic membranes is described.

Injection of rabbit serum of high anti-*Brucella* agglutinin titre into the lumen of one uterine horn resulted invariably in a significant positive agglutinin titre in the embryonic sera of all the normal embryos in it. No agglutinins could be detected in the sera of any of the embryos in the other or control uterine horn. No agglutinins could be detected in the maternal sera in eleven of the thirteen experiments, but the remaining two showed a low titre probably ascribable to faulty technique. It is shown that ligation of the vitelline vessels stops the circulation in the area vasculosa in the majority, but not in all embryos, the circulation being partially maintained in some, by anastomoses between the vitelline and allantoic

vessels or possibly restored by anastomoses around the ligature. The passage of agglutinins into the sera of these embryos depends on the maintenance of the vitelline circulation since no agglutinins could be detected in the embryonic sera of the majority, but agglutinins could be detected in a minority. The results show conclusively that agglutinins pass directly from the uterine lumen into the embryonic circulation of the area vasculosa.

Intravenous injection of immune serum into 24-day pregnant rabbits resulted in a positive titre in the sera of all the normal embryos. No agglutinins could be detected in the sera of half of the embryos in which the vitelline vessels had been ligatured, and the titres in the sera of the others tended to be lower than in the sera of the normal embryos. It was concluded that the allanto-chorionic placenta is an effective barrier to the passage of agglutinins in many, and probably in all, embryos. It was concluded also that agglutinins can pass from the maternal circulation into the uterine lumen on the 25th day of gestation. This latter conclusion was confirmed by the recovery of uterine fluid with a positive titre from a sterile uterine horn of one animal.

Samples of the exocoelomic, amniotic and allantoic fluids and of the stomach contents of the embryos were tested for agglutinins. Agglutinins were not detected in any of the samples from the embryos in the control uterine horns of the series of intra-uterine injections. Agglutinins were present in about half the tested samples of exocoelomic and amniotic fluids and of stomach contents of embryos exposed to immune serum. None were detected in any of the samples of allantoic fluid. The titre in the amniotic fluid never exceeded that in the exocoelomic fluid but did not appear to be correlated with that of the embryonic serum. These results suggest that agglutinins pass directly through the splanchnopleur into the exocoel and thence through the amnion. Individual idiosyncrasies may account for the irregularity of the results, in contrast to the regularity of passage into the embryonic blood. The occurrence and titre of agglutinins in the stomach contents and in the amniotic fluid appeared to be correlated, but the titre in the former tended to be higher. The occurrence and titre of agglutinins in the stomach contents and embryonic serum did not appear to be correlated. It is suggested that the agglutinins in the stomach contents are derived from the amniotic fluid, and tend to be concentrated in the stomach, either by differential absorption of water or by adsorption on mucus.

The significance of the results in relation to the structure of the placenta and embryonic membranes is discussed. It is suggested that the transitory permeability to antibodies of the gut of the new-born may be a neonatal extension of a characteristic antenatal property of the splanchnopleur. The possible value of the technique of intra-uterine injection during pregnancy as a means of introducing proteins into the embryonic circulation without interfering with the maternal circulation is indicated.

We are much indebted to Professor T. Dalling, M.A., M.R.C.V.S., for the interest he has taken in this work and for a supply of immune rabbit serum of high titre. We are greatly indebted also to Mr J. W. Newbiggin for much skilled technical

assistance. The work forms part of a larger scheme of research on prenatal mortality financed by a grant from the Agricultural Research Council to one of us (F.W.R.B.) for which we wish to express our thanks.

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# A discussion on antibiotic activity of growth factor analogues

OPENING REMARKS BY THE LEADER OF THE DISCUSSION

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GENERAL SURVEY

BY D. D. WOODS, *Department of Biochemistry, University of Oxford*

TYPES OF METABOLIC EFFECTS PRODUCED BY ANALOGUES

OF VITAMIN-LIKE SUBSTANCES

BY H. McILWAIN, *Research Laboratory, Maudsley Hospital, London*

SELECTIVE TOXICITY, AN ESSENTIAL REQUIREMENT FOR

SUCCESSFUL CHEMOTHERAPY

BY T. S. WORK, *National Institute for Medical Research, Hampstead, London*

THE DESIGN OF BACTERIAL INHIBITORS MODELLED ON GROWTH FACTORS

BY H. N. RYDON, *Department of Chemistry, Birkbeck College, London*

SOME ANTI-MALARIAL AGENTS AS POSSIBLE GROWTH-FACTOR ANTAGONISTS

BY F. L. ROSE, *Research Laboratories, Imperial Chemical Industries Ltd.,  
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GENERAL DISCUSSION

with contributions by

J. WALKER, *National Institute for Medical Research*

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M. STACEY, *University of Birmingham*

*(Discussion held 17 June 1948—Received 10 December 1948)*

OPENING REMARKS

BY SIR PAUL FILDES, F.R.S.

The subject of our discussion developed eight years ago as an offshoot of the study of bacterial nutrition, and it will be necessary for me to give a brief outline of this latter.

It is helpful to view the growth of bacteria as a purely chemical phenomenon. A bacterium is a congregation of enzymes and the proper co-ordinated working of these enzymes is reflected in an increase in mass which we call growth. By supplying a colon bacillus with the necessary chemical substrates it will increase its mass an English billionfold overnight. The necessary substrates are, of course, the elements which the bacterium contains and a source of energy. Among these elements is N in the form of  $\text{NH}_3$ .

We can therefore say that the colon bacillus grows by synthesizing its protein, including all enzymes and co-enzymes from  $\text{NH}_3$ .

Some information has been gained on how this is done. The synthesis proceeds in a series of steps, and each step is achieved by a synthetic enzyme capable of combining its two smaller substrates into one larger substance.

Taking as an example the biosynthesis of tryptophan, it is reasonably certain that the series of steps passes through anthranilic acid and indole to tryptophan, and that the second substrate of the enzyme which uses indole in the synthesis of tryptophan is serine. It is also reasonably certain that pyridoxal phosphate is a co-enzyme for this step. From the evidence, it appears that the mechanisms of these syntheses are common to most bacteria or even to other micro-organisms.

In some bacteria one of the enzymes in a chain is naturally lacking. In this case the synthesis is blocked, and the bacterium can only complete the synthesis and grow if it finds among its nutrients a component of the chain *above* the block. This substance is what the bacteriologist calls a growth factor. Any component of any synthetic chain is a growth factor for a bacterium which has lost the enzyme which should synthesize it.

If, therefore, we look upon growth as the realization of the proper working of a host of syntheses all proceeding step-wise and in complete co-ordination, we can appreciate that a blocking of any one of them will throw the whole process out of gear and growth will cease. As a working hypothesis and as a simplification we, indeed, suppose that any substance which inhibits bacterial growth, at any rate when the inhibition is reversible, does so by blocking some enzyme process necessary for the synthesis of bacterial protein.

In one or two cases the mechanism of blocking is reasonably well known:

(1). The inhibitor may react with a growth factor in such a way that it can no longer function. For instance, it is accepted that Hg and As react with the sulphhydryl group, a growth factor for bacteria, and so inactivate enzymes dependent on this group.

(2). We have the subject of the present discussion, i.e. the inhibitory action of analogues of growth factors.

The underlying idea here is that you can alter the structure of a growth factor so that it still fixes on its enzyme, but no longer acts as a normal substrate in combining with the second substrate of the enzyme. An analogue designed in this way, when added to a culture will compete with the real substrate of the enzyme, i.e. the growth factor, and, if in sufficient concentration, will block the enzyme. Biosynthesis and growth thus ceases. This will, of course, follow whether the growth factor is synthesized by the organism or added to the nutrients.

No doubt there are many other mechanisms by which bacterial growth may be inhibited. There seems to be little knowledge of the mode of action of the natural antibiotics like penicillin and so forth, but the recent work of Albert on the production of imbalance of trace metals by chelating agents opens a new field.

There is one further point I should like to make. This theory has been so productive in so many directions that it is a little surprising that nothing very useful in chemotherapy has come of it in eight years.

I think the reason is that these competitive reactions are delicately balanced and can only operate under highly controlled conditions.

If you design an analogue on the model of substance *A*, you readily obtain inhibition in the test-tube when you add a sufficient concentration of the analogue to compete with any of the substance *A* which the microbe can synthesize. In the fluid phases of animal blood however, where the bacteria are situated, there may be already such a concentration of *A* that you cannot compete with it and the bacteria will continue to grow.

Further, in the test-tube you may inhibit the use of substance *A* so that the next component *B* cannot be synthesized, but if you add mere traces of *B*, the competition with *A* is entirely ineffective in preventing growth. The synthesis continues from *B*. *B* may be present in the liquid phases of the blood. Thus, until you know the concentration of growth factors in the blood, your chances of selecting the most suitable growth factor as a model for your analogue are small. At present we have not this information.

Furthermore, on the chemical side, it seems desirable that more system should be used in the design of analogues. It is found that when one and the same substitution is made in different positions in a growth-factor molecule, the effect is quantitatively different. In this case it may be possible to deduce something about the mechanisms of the action. If one position only is taken haphazard you can deduce little.

Thus, greater knowledge of the physiology of blood, and greater knowledge of the mechanism of the action of an inhibitor, is required before it will be possible to design the right sort of analogue of the right sort of growth factor to give practical results.

#### GENERAL SURVEY

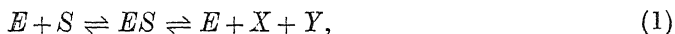
BY D. D. WOODS

#### *Introduction*

The object of the present communication is to give a broad outline of what is known concerning the antibiotic activity of growth-factor analogues, and to provide a background for the more detailed communications which follow. At the same time opportunity will be taken to fill in briefly unavoidable gaps between these communications; there are now so many aspects of the subject which have received attention that a full discussion would be impossible in the time available. For the same reason it will not be possible to give detailed references to the literature but only to reviews and certain selected recent papers.

The main concept under discussion is that the growth of micro-organisms may be inhibited by substances of related chemical constitution to factors which are essential for their growth. The discovery of the relationship of *p*-aminobenzoic acid (an essential metabolite) to the mode of action of the sulphonamides focused attention on this concept and has stimulated much new experimental work. The phenomenon of competitive inhibition of enzyme reactions by substances chemically related to the substrate was well established when the biological importance of *p*-aminobenzoic acid was first realized, and was, indeed, of fundamental importance for that

discovery. The classical example is that of the competitive inhibition by malonate of the oxidation of succinate by the succinic dehydrogenase enzyme system. The phenomenon is explained in terms of general accepted enzyme kinetic theory as follows:



An initial combination of the enzyme  $E$  and substrate  $S$  results in activation of the complex formed  $ES$  and is followed by decomposition of the complex with liberation of the enzyme and the products of substrate metabolism  $X, Y$ . The inhibitor  $M$  by virtue of its structural similarity to the substrate is envisaged as being able also to enter into combination with the enzyme. This combination does not, however, result in activation and consequently there is no decomposition of  $M$ . In the presence of both  $S$  and  $M$ , the overall rate of the enzyme reaction will thus depend on the relative rates of the reversible reactions (1) and (2), and therefore, for a given pair of compounds, upon their relative concentrations; the two substances, in effect, compete for the available enzyme (Haldane 1930).

#### *Mode of action of sulphonamides*

Extracts of many tissues are able to overcome the growth-inhibitory action of the sulphonamides. Strong evidence was obtained that the activity of yeast extracts in this respect is due to *p*-aminobenzoic acid, and this substance itself overcomes the effect of the sulphonamides in a competitive manner, i.e. there is a strict quantitative relationship between the concentration of drug used and the concentration of *p*-aminobenzoic acid required. This relationship, taken in conjunction with (*a*) the similarity in chemical structure between the antagonist and the parent drug, sulphanilamide, and (*b*) the high intrinsic activity of *p*-aminobenzoic acid (one molecule antagonizes *c.* 10,000 molecules of sulphanilamide), led directly to a working hypothesis concerning the fundamental mode of action of the sulphonamides. It was suggested that *p*-aminobenzoic acid is an essential substance for the growth of bacteria, and that sulphonamides, by virtue of their chemical similarity, inhibit competitively the enzyme system involved in the utilization of this substrate. In the terminology of the last section *p*-aminobenzoic acid would be  $S$  and the sulphonamides would be  $M$ . The hypothesis has received its most important confirmation from the fulfilment of the prediction that *p*-aminobenzoic acid is an essential metabolite for bacteria; it has been shown to be an essential growth factor (i.e. an external source is needed) for the growth of some fifteen different species of bacteria, yeast and moulds. Bacteria not needing an external supply are shown to synthesize it for themselves. The sulphonamide/*p*-aminobenzoic acid relation is of very general application both in respect to (*a*) the test organism and (*b*) the particular sulphonamide used (of the general type  $\text{NH}_2\text{-C}_6\text{H}_4\text{.SO}_2\text{NHR}$ ), the only exceptions being (*a*) *Bact. tularensis*, (*b*) sulphanilylanilides. This, and other evidence which cannot be dealt with here, provide more than sufficient support to warrant the retention of the hypothesis as a basis for further work. The form of the hypothesis outlined envisages *p*-aminobenzoic acid as the substrate of the enzyme; similar relationships would, of



course, also exist if this substance were a co-enzyme or prosthetic group whose combination with the enzyme was subject to competition by sulphonamides.

For a fuller understanding of the biochemical mode of action of the sulphonamides the next step is to discover the nature of the products of the reaction in which *p*-aminobenzoic acid is involved either as substrate or possibly as co-enzyme. Considerable evidence is now to hand that *p*-aminobenzoic acid functions either directly or indirectly in the biosynthesis of (a) folic acid, (b) purines, (c) the pyrimidine, thymine and (d) certain amino-acids (methionine, serine, lysine and possibly others). In the case of folic acid the function is direct; in the other cases it appears to be indirect, and evidence for these functions is based largely on findings that these substances, either singly or in various mixtures, may replace *p*-aminobenzoic acid, either wholly or in part, both in respect of its function as a growth factor and as an anti-sulphonamide agent. There is some evidence (from replacement experiments with organisms requiring folic acid) that the function in the synthesis of the nucleic acid derivatives occurs through folic acid. If all the substances in whose synthesis *p*-aminobenzoic acid is concerned are provided preformed in the medium, two things would be expected (provided the substances can be absorbed by the cell): (a) organisms requiring *p*-aminobenzoic acid should grow in the absence of this factor, (b) all organisms should be insensitive to sulphonamides since the products of the inhibited reactions have been already given.

Folic acid is another essential metabolite (one of the vitamin B group of factors) both for micro-organisms and for higher animals (see review by Jukes & Stokstad 1948). A direct role of *p*-aminobenzoic acid in the biosynthesis of this factor has been made clear by the elucidation of the structure, and synthesis of, one member of the folic acid group of vitamins (*Lactobacillus casei* factor). This compound (pteroyl-glutamic acid) is *N*-[4-[[[(2-amino-4-hydroxy-6-pteridyl) methyl] amino] benzoyl] glutamic acid; it thus contains a *p*-aminobenzoic acid residue linked through the amino-group to a pteridin residue and through the carboxylic-group to glutamic acid. It is therefore likely that *p*-aminobenzoic acid is one of the substances required for its biosynthesis. There is now direct evidence that the synthesis of *Lb. casei* factor by cells of *Lb. plantarum*, under conditions in which no growth occurs, depends quantitatively on the presence of *p*-aminobenzoic acid, and that the synthesis is inhibited competitively by the sulphonamides.

With some organisms there is good evidence that the only function of *p*-aminobenzoic acid may be its requirement for folic acid synthesis, and that inhibition of this reaction represents the main point of attack of sulphonamides on the cell. Organisms requiring folic acid itself as growth factor have been found to be almost insusceptible to inhibition by sulphonamides; this would be expected, since the metabolic lesion presumed to be induced by sulphonamides already exists and the product of the inhibited reaction must be added to obtain growth even without sulphonamides. Certain enterococci for which folic acid is stimulatory though not essential (indicating that the rate of synthesis of this factor is limiting growth) are highly susceptible to sulphonamides in the absence of folic acid, but again almost completely insensitive in its presence. Finally, with some organisms (but not all) which require *p*-aminobenzoic acid for growth it has been found that pteroyl-

glutamic acid can replace this factor, although a higher molar concentration is required, and that growth is not inhibited by sulphonamides over a wide range of concentration in the presence of pteroylglutamic acid at the concentration in which it is effective in replacing *p*-aminobenzoic acid for growth. It should be remarked that in many cases (though not all) the basal media for the above organisms contained nucleic acid derivatives and amino-acids, since these are known to be essential for some of the organisms irrespective of the requirement for *p*-aminobenzoic acid or folic acid.

With regard to other organisms that have been tested (e.g. several other species for which *p*-aminobenzoic acid is a growth factor, also several species not requiring an external source of this factor or of folic acid) the position is apparently quite different. Synthetic pteroylglutamic acid appears to act only as an inefficient source of *p*-aminobenzoic acid; sulphonamide inhibition is antagonized, if at all, in a competitive manner. This matter cannot be considered in detail here, but it may be said that there is evidence, from observations of the growth factor and anti-sulphonamide action of various mixtures of nucleic acid derivatives, amino-acids and pteroylglutamic acid, that in such organisms *p*-aminobenzoic acid may function indirectly in the synthesis of all these substances, and that all these functions are subject to inhibition by sulphonamides. It cannot yet be decided whether the mechanism is quite different in these various syntheses or whether folic acids not identical with synthetic pteroylglutamic acid may be intermediates.

For full details of the work described in this section reference may be made to the reviews of Henry (1944), Roblin (1946), Woolley (1947), Work & Work (1948) and Woods (1949).

The therapeutic value of the sulphonamides is due to their toxicity to the invading microbes and relative lack of toxicity to the host. The work outlined above provides some explanation for this difference. Folic acid is also an essential metabolite for mammals, and the available evidence indicates that no simpler compound than folic acid will serve. The animal therefore resembles those bacteria which require preformed folic acid and would be relatively insensitive to sulphonamides for the same reasons. This matter is discussed in more detail by Woolley (1947) and by Woods & Nimmo-Smith (1949).

#### *A rational approach to research in chemotherapy*

The discovery of the relationship between *p*-aminobenzoic acid and sulphonamides, providing as it did a rational explanation in biochemical terms of the mode of action of these drugs, stimulated a search for analogues of other known bacterial growth factors which might inhibit growth for the same reasons. This concept was enunciated by Fildes (1940) in his paper on 'a rational approach to research in chemotherapy'. Since then a very large number of substances of this type have been found which inhibit specifically the growth of micro-organisms, i.e. their action is overcome by the growth factor on which they are modelled (see reviews by Roblin (1946) and Woolley (1947)). Only very few, however, have shown any promise of being useful therapeutic agents, and these are all analogues of pantothenic acid—a point that merits further research. It should be emphasized that

this approach can only predict substances likely to have anti-bacterial activity; it cannot predict, for example, whether they will be suitable from the point of view of absorption, excretion or possibly destruction by the host tissues. Another difficulty is that success as a therapeutic agent implies selective toxicity against the invading microbe. Many of the compounds made have been analogues of substances (B-group vitamins, amino-acids) which are well known to be equally essential for the metabolism of host tissues; indeed, these analogues have often been found to induce in the host specific deficiency symptoms of the vitamin in question. This is a major difficulty in view of the fundamental similarity of basic reactions in all types of cells. What are needed are analogues of factors essential only for bacterial growth, and Dr Work will suggest later an interesting and promising approach to this problem. Another possible solution which has been suggested (Roblin 1946) is to concentrate upon analogues of factors (as these become known) which are concerned with anabolic processes of the cell. Even if these are not selective against bacteria it is possible that a short-term interruption to such processes may do relatively little harm to the adult host in which rapid cell proliferation occurs in a few tissues only; at the same time bacterial growth might be held in check sufficiently long to permit the normal defence mechanisms of the host to deal with the situation.

The recent studies on the *p*-aminobenzoic acid/sulphonamide relationship make it clear that a detailed knowledge of the function of a growth factor in cell metabolism may be of considerable aid in evaluating the possible therapeutic value of the analogue. Dr McIlwain will review our knowledge concerning the point of action of some other analogue inhibitors in cell metabolism.

Finally, it must be realized that in the past the design of these growth inhibitors has often been based on rather 'pictorial' considerations of the chemical structure of the growth factor and related substances. Dr Rydon will illustrate the value of more fundamental considerations of chemical structure, especially in relation to what specific chemical grouping are involved in the union of the substrate with the enzyme.

#### *Possible anti-metabolite action of other growth inhibitors*

There is some evidence that the activity of a few other substances which inhibit growth of micro-organisms (including two natural antibiotics) may be due to competition with metabolites. It must be strongly emphasized that the evidence is never more than suggestive, and that the cases quoted must be considered at the present only as interesting possibilities.

Dr Rose will examine such possibilities in the case of some of the anti-malarial drugs, including paludrine. Another synthetic drug which has been considered as possibly acting in this way is the insecticide 'Gammexane' ( $\gamma$ -hexachlorocyclohexane). Slade (1945) pointed out that the stereochemical configuration of the  $\gamma$ -isomer was probably analogous to that of meso-inositol (which is known to be a growth factor for yeast and other micro-organisms), and he suggested that 'Gammexane' may act on insects by an effect on inositol metabolism. This idea received support from the observation of Kirkwood & Phillips (1946) that 'Gammexane', but not the other isomers, inhibited the growth of a yeast which required

inositol, and that such inhibition was overcome by increasing the inositol concentration in the medium. But this is not the case with all micro-organisms; with *Tetrahymena geleii* and with a number of bacteria it has been found that  $\delta$ -hexachlorocyclohexane is more inhibitory than the  $\gamma$ -isomer, and that in neither case is inhibition overcome by inositol (Chaix, Lacroix & Fromageot 1948; Fromageot & Confino 1948).\*

There is an interesting observation of Rhymer, Wallace, Byers & Carter (1947) which suggested to them the possibility of an anti-metabolite activity of streptomycin. Inhibition of the growth of *Staphylococcus aureus* was overcome by purified preparations of lipositol; the amount needed was in the proportion 1 lipositol/300 streptomycin. Lipositol is a phospholipoid obtained first from brain and later from soy-bean; its structure is not yet fully worked out, but it is an inositol derivative containing, amongst other substituents, a galactose residue (Woolley 1943). Streptomycin is also an inositol derivative containing carbohydrate residues. There is, of course, no evidence that lipositol is an essential metabolite for bacteria, but only that it is of natural occurrence; its detection in nerve tissue is of interest in view of the toxic action of streptomycin on the VIIIth nerve.

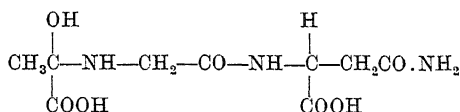
Lycomarasmin is an antibiotic produced by *Fusarium lycopersici*, and is responsible for the tomato-wilt effect of this fungus; it also inhibits the growth of bacteria. Woolley (for references see Woolley 1948a) followed up earlier work on the chemical nature of this compound and found that seryl-glycyl-aspartic acid had considerable lycomarasmin activity. At the same time he and his collaborators were working on a growth stimulant (strepogenin) for certain streptococci and lactobacilli, and obtained evidence that it was probably a peptide containing glutamic acid. Concentrates overcame the antibiotic effect of lycomarasmin on both tomato plants and bacteria. Applying the analogue-inhibitor concept Woolley therefore synthesized (amongst other peptides) seryl-glycyl-glutamic acid. This had strepogenin activity in all respects though it cannot be strepogenin itself, since it had only 2.5 % of the activity of the most purified preparations of the natural factor.†

#### *Metabolite analogues as tools in fundamental research*

In the case of lycomarasmin just discussed the concept of inhibition by growth-factor analogues has led to some valuable information concerning the chemistry of the growth factor concerned. It would be a pity to consider these analogue inhibitors only as potential therapeutic agents; they have also proved to be valuable tools in

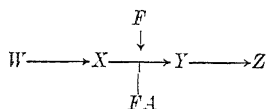
\* Since this discussion was held, van Vloten, Kruissink, Strijk & Bijvoet (1948) have shown by an X-ray analysis of the crystal structure of 'Gammexane' that it is not isomorphous with meso-inositol.

† Since this discussion was held Woolley (1948b) has obtained strong evidence that the structure of lycomarasmin is



The synthesis and testing for strepogenin activity of the corresponding analogue containing a glutamine rather than an asparagine residue will be of great interest.

helping to elucidate the function of growth factors and vitamins in cell metabolism. They act as rather specific inhibitors of the utilization or synthesis of the analogous factor and thus provide a means of tracing the primary effect of that factor. Suppose that the biosynthesis of some essential substance  $Z$  occurs in the following stages:



and that a factor  $F$  is involved in one of these stages. Competitive inhibition of the function of  $F$  by an analogue  $FA$  will block the reaction sequence and growth will not occur. Theoretically inhibition may be overcome by adding an extra supply of  $F$  or by giving the products ( $Y$  or  $Z$ ) which are beyond the block. One example only must serve to illustrate this point. Shive & Rogers (1947) have found that 3, 4-urylenecyclohexylbutyric acid, an analogue of biotin, inhibits the growth of *Lactobacillus arabinosus* (an organism requiring biotin). Inhibition is reversed competitively by biotin, thus establishing the specificity of the effect. The amount of biotin required is, however, reduced tenfold if oxaloacetic acid is present, and this suggests that one function of biotin is concerned with the synthesis of this keto-acid; evidence derived from other studies indicates that this synthesis probably occurs through the condensation of  $\text{CO}_2$  and pyruvic acid.

#### *Analogue inhibitors as growth factors*

It sometimes happens that a given substance may act as growth inhibitor for one organism and as growth factor for another organism or variant of the same organism. Two such cases may be quoted from the growth-factor analogues; in one case there is a simple explanation, in the other, not. Desthiobiotin can replace biotin for the growth of yeast; the organism can complete the synthesis from this compound, which may indeed be a normal intermediate, the requirement of such yeasts for biotin being due to a failure at an earlier stage of the reaction sequence. *Lb. casei*, on the other hand, is unable to convert desthiobiotin to biotin, and here the former acts as an inhibitory analogue, prevents the utilization of biotin and thus suppresses growth.

Emerson (1947) has studied some interesting variants of *Neurospora*. One of these, obtained by crossing a sulphonamide-resistant strain with the wild type, actually has an essential requirement for sulphonamide for growth at temperatures above  $30^\circ$ ; the effect is less marked at lower temperatures. It seems probable that *p*-aminobenzoic acid remains an essential metabolite; by crossing this variant with a mutant requiring *p*-aminobenzoic acid a variant was obtained which requires both this substance and sulphonamide. *p*-Aminobenzoic acid inhibits the sulphonamide-promoted growth of these organisms, but it is interesting that the critical ratio between the two substances remains the same whichever is acting as growth factor.

#### *Miscellaneous*

One further interesting application of the general concept of biological antagonism between analogues may be mentioned. Green & Woolley (1947) followed up a suggestion of Hirst (1942) that the agglutination of red cells by influenza virus may be

due to an enzymic attack on a specific substrate, probably of a polysaccharide nature, in the cell. They therefore tried the effect of a number of polysaccharides on the reaction. Several of these, particularly apple pectin, inhibited haemagglutination; furthermore, the apple pectin also inhibited multiplication of the virus in egg embryo.

In conclusion, it should be stressed that a complete understanding of the function of individual essential metabolites in cell metabolism, and of the comparative specificity of requirement by host and micro-organism, is one of the essentials for a profitable exploitation of growth-factor analogues as potential chemotherapeutic agents. And the analogues themselves are proving to be valuable tools in such fundamental investigations whether or not they are themselves useful as therapeutic agents.

#### TYPES OF METABOLIC EFFECTS PRODUCED BY ANALOGUES OF VITAMIN-LIKE SUBSTANCES

BY H. McILWAIN

##### *Functional relations between analogue and growth factor*

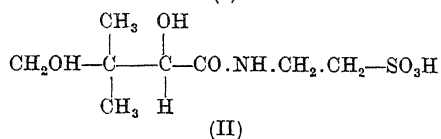
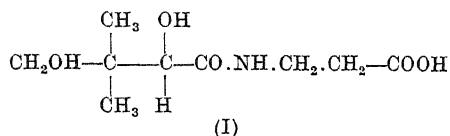
Evidence connecting the action of an analogue with its parent growth factor is of three main types.

##### *I. Antagonism in action*

This can be the clearest of all types of evidence; it has been much discussed, classified, and subjected to mathematical expression. For that reason I will say most about other types of evidence, and make only two observations concerning this first category. (a) The antagonism can be observed at various levels: that of an overall biological effect such as growth, or at that of an individual enzymic

TABLE 1. SYSTEMS IN WHICH ANTAGONISM BETWEEN PANTOTHENIC ACID (I)  
AND PANTOYLTAURINE (II) HAS BEEN OBSERVED

1. Chemotherapeutic: protection of infected rat from haemolytic streptococci.
2. Microbiological: streptococcal growth.
3. A series of enzyme reactions: resulting in assimilation of pantothenate to growing streptococci.
4. A simpler enzyme system: resulting in inactivation of pantothenate by non-proliferating streptococci during glycolysis.
5. (Interaction at a simpler system may be anticipated.)



process in which the growth factor takes part. Such observations can be employed in understanding the effect on growth, in terms of events in simpler systems (table 1; McIlwain 1947). (b) There are varying degrees of specificity in the antagonistic relationships between a given inhibitor and growth factors. In the simplest case, only one growth factor may be recognized as antagonist; as is pantothenate to the action of pantooyltaurine on streptococci. In other cases, the types of antagonist can give clues to the action of the antibiotic; as with *p*-aminobenzoic acid, *Lactobacillus casei* factor, and the pyrimidines which antagonize sulphonamides. Such multiple antagonists can give clues to the nature of the systems affected by the analogues.

## II. *Specificity of action*

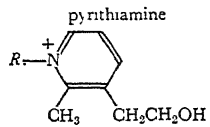
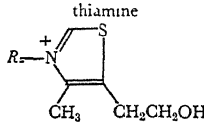
In several instances a correlation is found between the species of organisms which require a given substance for growth, and the species which are susceptible to inhibition by a given antibiotic. An instance is given by Woolley (1945-6) of the use of such data as evidence for functional connexion between thiamine and pyriethiamine (for structure see table 2). Similar correlations have been found in the actions of pantooyltaurine on different microbial species, and their need for pantothenic acid (McIlwain 1942). Other analogues have, however, a range of action which includes organisms which do not require preformed the corresponding growth essential. Of this category are sulphanilamide and phenylpantothenone (Woolley 1945). Findings such as those with pyriethiamine or pantooyltaurine present interesting problems because they suggest that the more exacting organisms possess processes (susceptible to inhibition) additional to those of the less exacting strains. The reasons for the correlation in such cases are not clear, and may not be the same in the different cases. Production of a large excess of growth factor by the less exacting strains has not been found to offer an explanation in the instances quoted above. Organisms requiring a substance as growth factor may differ from those synthesizing the substance, in possessing specific means of assimilating it which are susceptible to inhibition. This would imply evolution to more exacting organisms to be primarily conditioned by gain, and not by loss, of function. Also, the route through which a higher, functioning derivative of the growth factor is synthesized may not involve the free growth factor. A further possibility is of metabolic change in the analogue.

## III. *Characters of the action of the analogue*

Evidence of this category has been adduced mainly in connexion with the actions of analogues on higher animals. Here Woolley's work is outstanding. He has assessed the changes in form and behaviour of experimental animals following administration of the analogues listed in table 2, and observed changes similar to those of the corresponding avitaminoses. There is scope for similar work with many other organisms; insects, plants, and even micro-organisms. This is very much a challenge to microbiologists to learn more about changes which precede total loss of function. Thus, changes in protein make-up may be determined by immunological methods. Changes in the form of bacterial colonies and cells, or of their capsules

(such as have been observed to follow nutritional changes), could be used as criteria for comparing the effects of withdrawing a nutrient with that of adding an analogue. The type and rate of bacterial growth can also be followed in much more detail than is commonly observed, and abnormal metabolites may be observed to be produced in the presence of subinhibitory concentrations of antibiotics (Fox 1942; Stettin & Fox 1945). Such changes may give very direct clues to the action of the analogue; in at least one instance, that of biotin and biotin sulphone, the growth factor itself was observed to be released by the analogue from its combination with a protein.

TABLE 2. ANALOGUES OBSERVED TO PRODUCE SYMPTOMS SIMILAR TO THOSE CAUSED BY LACK OF THE CORRESPONDING GROWTH ESSENTIAL

analogue	growth factor	symptoms in mice
glucoascorbic acid, $R \cdot \text{CHOH} \cdot \text{CH}_2\text{OH}$	ascorbic acid $R \cdot \text{CH}_2\text{OH}$	lesions similar to those of scorbutic animals
 3-acetyl pyridine, $R \cdot \text{COCH}_3$	 nicotinic acid, $R \cdot \text{COOH}$	hyperirritability, anorexia, convulsions, characteristic posture
		respiratory changes, loss of control of limbs, epidermal changes, emaciation

#### *Classification of the actions of analogues*

In the majority of recorded instances, therefore, the actions of analogues of growth factors can be seen to be connected in some way with that of the growth factor itself. Now the growth factors of which we are speaking are chemical substances; after growth of organisms, necessarily in their presence, the substances are found in the cells or tissues of the organism concerned, either as such or in combination. Very often they can be observed there to be both undergoing and influencing further chemical changes.

These very general observations give the basis for a classification of the action of the analogues: they may effect either (1) assimilation of the growth factor (2) steps in its conversion to a functioning form or (3) its functioning.

#### *(1), (2) Assimilation and synthesis*

In some instances no basis may exist for differentiation between these two stages; in other cases they are known to be distinct. Only rarely have serious attempts been made to differentiate between the two processes in relation to the action of analogues. Assimilation of a growth essential is often a considerable process; nutrient or tissue fluids may contain nicotinic acid, thiamine or riboflavin in concentrations of  $10^{-6}$  to  $10^{-7}$  M, and tissue cells or micro-organisms contain them or their derivatives at  $10^{-3}$  or  $10^{-4}$  M. Active and specific processes are necessary to bring about such concentration; for only certain substances are removed from the environment of cells in this way. But the processes causing assimilation may not be



distinct from those which cause chemical elaboration of the growth essentials to the forms in which they function. Thus, almost all the nicotinamide of bacterial cells exists as nucleotides. To determine whether there existed, for example, a process for the assimilation of nicotinamide which was distinct from that forming the (pyridine N)-C link of co-enzymes I or II, would require specific evidence, such as the measurement of free nicotinamide within and without cells in which that process had been inhibited. Such observations do not appear to have been made. It might seem possible to obtain data on assimilation of the co-enzymes themselves by comparable measurements, as they certainly exist as such in cells; but here we find that they are rapidly broken down if added (McIlwain & Hughes 1948); and in no instance in which investigations have been carried out have the co-enzymes proved to constitute an organism's minimal requirements for nicotinamide derivatives.

TABLE 3

$\begin{array}{c} \text{COOH} \\   \\ \text{CH}-\text{NH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{SO} \\   \\ \text{CH}_3 \end{array}$	$\begin{array}{c} \text{COOH} \\   \\ \text{CH}-\text{NH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CO} \\   \\ \text{OH} \end{array}$	$\begin{array}{c} \text{COOH} \\   \\ \text{CH}-\text{NH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CO} \\   \\ \text{NH}_2 \end{array}$	$\begin{array}{c} \text{COOH} \\   \\ \text{CH}-\text{NH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{CO} \\   \\ \text{NHNH}_2 \end{array}$
sulphoxide derived from methionine	glutamic acid	glutamine	$\gamma$ -glutamyl hydrazine

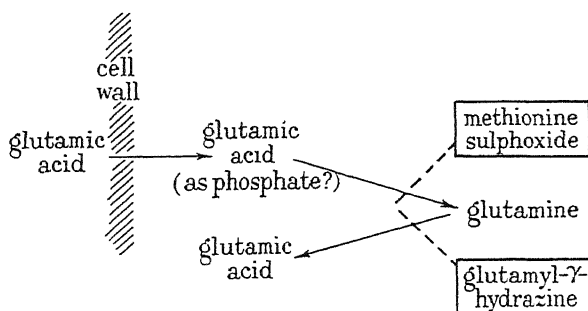


FIGURE 1. Streptococcal metabolism of glutamic acid, and its inhibition by analogues. (McIlwain *et al.* 1948; each stage is associated with energy-yielding reactions).

Methionine sulphoxide (cf. Borek, Miller, Scheiness & Waelsch 1946; Waelsch, Owades, Miller & Borek 1946), which inhibits the growth of streptococci by processes shown by methods of § I above to be related to the formation of glutamine, has been investigated from this point of view (McIlwain, Roper & Hughes 1948; Roper & McIlwain 1948; for formulae see table 3). Reactions in which glutamic acid is known to take part in these organisms are shown in figure 1. The formation of glutamine could be prevented either by stopping the entry of glutamic acid to the cells, or by stopping the formation of glutamine from glutamic acid after it had entered. We actually found that a concentration of sulphoxide which

lowered the synthesized glutamine of streptococci from 0.70 to 0.35  $\mu\text{mol./g.}$  lowered the entry of glutamic acid only from 30 to 25  $\mu\text{mol./g.}$  The sulphoxide therefore appeared to act after assimilation of glutamic acid.

Though differentiation between the action of analogues on assimilation and synthesis may only rarely have been realized, action on either of the two processes results in a lesser quantity of functioning growth factor in the cells concerned, and this can be readily differentiated from the third category in which action of an analogue is on the functioning of a growth factor. Sulphanilamide and pantoyltaurine do not appear to influence the functioning of the corresponding growth factors when once these are already in action in the cell. Several characteristics of the effects of the compounds on microbial growth can be seen to result from this specificity in their point of action; the latent period before their effect on growth is manifest; their bacteriostatic, not bactericidal action, and the absence of actual displacement of the growth factor from the cells, by the analogue (McIlwain 1945).

### (3) *Functioning*

Growth essentials subserve many types of function in cells. Glucose, oxygen, or some amino-acids are found to be taking part in major reactions as sources of material and energy; nicotinamide, aneurin, or pantothenic acid as parts of systems by which changes of the first category are brought about.

Analogues of oxygen are, of course, among the most potent of antibiotics. Carbon monoxide and cyanides can cause death in a few seconds when present in concentrations only a fraction of that of the oxygen with which they compete. They exhibit a specificity of action which in the case of cyanides is applied commercially on a large scale, and their success should be a challenge and a stimulus to those attempting to produce analogues of biological interest.

There are a few instances in which analogues of substances subserving the latter catalytic type of function have been demonstrated to act at the point of catalysis. It has been reported that pyridine-3-sulphonic acid inhibited glucose oxidation, in which the nicotinamide nucleotides functioned (Euler 1942), but no attempts were made to correlate this effect with the antibiotic action of the substance. Such evidence is, however, available in the case of the actions of glutamine and the corresponding hydrazide on streptococcal growth. The action of the hydrazide was shown by methods of §§ I and II to be related to glutamine, but its action proved to be distinct from that of the sulphoxide derived from methionine (figure 1); it did not affect the assimilation of glutamic acid or the synthesis of glutamine, but it inhibited the breakdown of glutamine to glutamic acid and  $\text{NH}_3$ , a process which was associated with increase in glycolysis. The inhibition of growth was correlated with the inhibition of breakdown of glutamine. There is also evidence suggesting that glucoascorbic acid inhibits the oxidation of tyrosine by ascorbic acid (cf. Woolley 1946).

It will be seen from this survey that serious study of the point of action of growth-factor analogues has been barely begun. It is, however, very necessary that the subject should be pursued if correlations are attempted between the biological effects of analogues, and their chemical structures. The actions of the sulphoxide

derived from methionine and of glutamyl hydrazine, though both antagonized by glutamine, are biochemically distinct. Localization of the point of action of an analogue, as illustrated in figure 1, is thus an essential preliminary to any interpretation of their potencies in electromeric terms.

SELECTIVE TOXICITY, AN ESSENTIAL REQUIREMENT FOR  
SUCCESSFUL CHEMOTHERAPY

BY T. S. WORK

Chemotherapeutic research is judged, ultimately, by its ability to produce new and successful drugs for the treatment of disease. Success may be fortuitous or it may be the result of an inspired guess into the future. It is my task to-day to examine current theories on metabolite analogues and to assess their value as a pointer towards further progress.

A glance at past progress may help us to assess more fairly the present position. Table 4 gives a short list of notable chemotherapeutic drugs together with their origin and date of introduction into European medicine.

TABLE 4. HISTORICAL PROGRESS OF CHEMOTHERAPY

drug	date introduction European medicine	remarks
santonin	before A.D. 300	European folk medicine
quinine	1633	borrowed from Americas
ipecacuanha	1658	borrowed from Americas
chaulmoogra	1854	borrowed from India
atoxyl	1905	Thomas
trypan blue	1906	Mesnil & Nicolle
salvarsan	1912	Ehrlich & Bertheim
stibenyl	1916	Caronia
tryparsamide	1919	Jacobs & Heidelberger
Bayer 205	1920	Bayer, A. G.
plasmoquine	1924	Schulemann <i>et al.</i>
atebrin	1933	Mauss & Mietzsch
sulphanilamide	1936	Tréfouël <i>et al.</i>
sulphapyridine	1938	May & Baker Ltd.
penicillin	1941	Chain, Florey and others
paludrine	1946	Curd & Rose

It is apparent from this table that shortly before 1900 prevailing ideas must have undergone some fundamental change which resulted in a sudden blossoming of the whole subject. The new idea was the germ theory of disease, and its direct offshoot was the subculture of pathogenic organisms both *in vitro* and *in vivo*.

Despite the accumulation of information for over forty years there has been no marked acceleration of the rate of progress, and the production of a new and successful drug is still a long and difficult problem. Indeed, so much is this true, that a prominent research worker in the field recently remarked that he knew only two methods for the production of new drugs—‘the hit-and-miss method, where the

starting point was a compound of known activity upon which the organic chemist played variations, and the miss-and-miss method, where the starting point was any old chemical from the store cupboard'. With this criticism in mind, I should like to review some of the theoretical and practical achievements of recent years.

The first general theory of chemotherapy, Ehrlich's receptor theory, did much to stimulate the early stages of chemotherapeutic research, but rather surprisingly failed to associate receptors with enzymes. The idea was not wholly ignored, however, as Simon & Wood (1914) remarked, 'since intracellular metabolism is intimately connected with the action of enzymes the question has naturally suggested itself whether the deleterious action of dyes may not in part be referable to interference with the activity of these components'. Over a period of years, the idea that drugs act through enzyme inhibition gained gradual acceptance and finally crystallized in one form as the Fildes-Woods hypothesis, upon which I need not expand. I must, however, draw attention to the comparative failure of research based on the production of metabolite analogues to provide any significant new advance in practical chemotherapy.

Those outstanding anti-metabolites, the sulphonamides, were discovered before the recognition of the metabolic importance of *p*-aminobenzoic acid and must be credited to the account of the old hit-and-miss method. The thiamin analogue pyriethiamin inhibits certain bacteria, but is useless as a chemotherapeutic remedy because it causes thiamin deficiency in the host (Woolley & White 1943). The pantothenic acid analogue, pantoyletaurine, shows slight chemotherapeutic activity, but is over-rapidly excreted and also inactivated by pantothenic acid of the host tissue (McIlwain & Hawking 1943). One minor success of the anti-metabolite approach is the treatment of malaria by phenylpantothenone and other pantothenic acid analogues (Woolley & Collyer 1945; Brackett, Waletzky & Baker 1946; Seneac, Rapport & Koepfli 1947). This comparative failure should be contrasted with the success of the more orthodox approach to chemotherapy which has provided in the same period, 1940 to 1948, such drugs as penicillin and paludrine, to mention only two out of many.

Should we, then, abandon the anti-metabolite approach as philosophically attractive but useless in practical chemotherapy? To do so would surely be to display excessive impatience. In the brief period of eight years since its inception this approach has, as already mentioned, produced one series of drugs active in clinical malaria. But for quinine and the armament of synthetic anti-malarials collected during more than forty years of intensive research, these compounds would have been hailed as a considerable triumph for the anti-metabolite theory. Perhaps it is not so much the theory which is at fault, but rather that we expect too much of it and know too little to apply it. Our theoretical approach to the synthesis of new drugs *may* require modification, our knowledge of cellular metabolism *certainly* requires expansion.

Study of the mode of action of certain successful chemotherapeutic drugs suggests one direction in which the anti-metabolite approach might be advantageously modified. As long ago as 1909, Ehrlich suggested that arsenicals might be toxic because of their affinity for thiol groups. He and his contemporaries proceeded to

develop one of the most successful groups of drugs, the organic arsenicals, but they failed to suggest any reason why these drugs destroyed the pathogen rather than the host. This difficulty was clearly recognized by Voegtlin (1925), who remarks, in his discussion: 'we agree with this explanation as far as it goes, but it still has to be explained why the host tissues can resist the toxic action of a slow stream of arsenoxide better than the parasites.' At another point in the same paper Voegtlin says: 'a vast amount of work on the biochemistry of both host tissues and especially of the parasites is required to settle this point and new experimental methods must be devised for the solution of this fundamental problem.' Voegtlin's plea has not gone unanswered; with the growth of enzymology and parasitology new experimental methods have been devised, and the results obtained do suggest an explanation for the selective toxicity of arsenicals.

In 1929 Yorke, Adams & Murgatroyd developed a medium for the maintenance of pathogenic trypanosomes and showed that *Trypanosoma rhodesiense* consumed more than twice its own weight of glucose in 24 hr. The presence of glucose was essential for the life of the trypanosome; glucose consumption and respiration were extinguished simultaneously by trypanocidal arsenoxides. The inference drawn from these observations was that trypanocidal drugs acted by inhibiting glucose metabolism.

The nature of glucose metabolism in trypanosomes has been indicated by various observations extending over a number of years. Reiner & Smythe (1934) showed that *T. equiperdum* produced pyruvic acid from glucose; Fulton & Stevens (1945) found pyruvic acid, lactic acid, acetic acid, formic acid, succinic acid, glycerol and ethanol as products of metabolism in *T. rhodesiense*; Chen & Geiling (1946) showed that lysed trypanosomes converted glucose to fructose-1:6-diphosphate, and this to 3-phosphoglyceraldehyde and to phosphoglyceric acid. Glucose metabolism of *T. rhodesiense* was not inhibited by cyanide (Christophers & Fulton 1938). All these results taken together suggest that in some strains of pathogenic trypanosome glucose metabolism follows the anaerobic glycolytic pathway mapped out for muscle and for yeast fermentation. Certain enzymes of this system have been shown to be SH-enzymes and are readily inhibited by extremely low concentrations of SH-reagents such as iodoacetamide, lewisite and the various arsenoxides (Barron & Singer 1945; Dixon & Needham 1946). At first sight it may seem surprising that an inhibitor such as phenylarsenoxide should owe its selective toxicity to inhibition of an enzyme system common to host and parasite, and several workers have fallen back upon the last line of defence—selective distribution.

Permeability and distribution theories are always difficult to prove and almost impossible to disprove and are therefore a favourite refuge from a difficult situation, but in this case a number of observations suggest that there is no call for retreat to such a refuge. No doubt some of the differences between individual arsenoxides can be attributed to differences of distribution, but it seems improbable that such widely different types of arsenical as arsenious acid, atoxyl and arsenophenylbutyric acid could all owe their ability to destroy trypanosomes selectively, in the presence of host cells, to a favourable distribution ratio. Moreover, if distribution were the key to the problem it would be most surprising that no organic arsenical

can be devised which will selectively destroy *T. cruzi*. Selective inhibition of an enzyme system of vital importance to the pathogen and less vitally important to the host seems to be the preferable explanation.

The arsenic sensitive group of trypanosomes have a high rate of cyanide-insensitive glucose metabolism and are susceptible to selective destruction by arsenicals. Other trypanosomes differ from this group in that their glucose metabolism is highly sensitive to inhibition by cyanide; they cannot be selectively destroyed in the presence of host cells by organic arsenicals (von Brand, Johnson & Rees 1946; von Brand & Tobie 1948). Animals also possess a cyanide-sensitive respiratory system and can probably oxidize glucose in the presence of inhibitors of glycolysis by an alternative metabolic pathway involving phosphogluconic acid (Dickens 1938).

It seems a reasonable assumption, then, that organic arsenicals are effective in eliminating certain trypanosome infections because the trypanosomes concerned differ metabolically from the host cells in being entirely dependent upon a single mechanism of energy production, glycolysis, which is readily inhibited by arsenoxides. This view is supported by the observations of Marshall (1948). *T. evansi*, an arsenic-sensitive trypanosome, is dependent for energy supply on the metabolism of glucose to pyruvate and is unable to utilize other common metabolites such as succinate, fumarate or amino-acids. In this respect it differs markedly from the host cells. Iodoacetate, a specific inhibitor of SH-enzymes, inhibits respiration of *T. evansi* to the extent of over 90 % at a concentration of M/4000; the same inhibitor at a concentration of M/3000 inhibits *T. cruzi* respiration only to the extent of 13 % (Marshall 1948; von Brand *et al.* 1946). The metabolic pathways obviously differ in the two types of cell.

Detailed study of the mode of action of sulphonamides has indicated, as pointed out by Dr Woods at this meeting, that these drugs also owe their selective toxicity to their ability to inhibit a metabolic reaction (formation of pteroylglutamic acid) which is essential to sulphonamide-sensitive organisms but not so essential to the host.

Penicillin is the most outstanding example of selective toxicity among chemotherapeutic drugs. It is highly toxic to a large group of Gram-positive organisms and practically non-toxic to Gram-negative organisms or to animal cells. Gale & Taylor have pointed out that Gram-positive organisms differ from Gram-negative in that they require a number of preformed amino-acids in their medium. Glutamic acid is synthesized readily by Gram-negative organisms and by animals, but has to be supplied in the medium of penicillin-sensitive Gram-positive organisms. It is transported across the cell wall of these organisms by an energy requiring metabolic reaction which is absent from Gram-negative micro-organisms (Gale & Taylor 1946, 1947; Taylor 1947). A strong case has been made out by Gale that the selective toxicity of penicillin is due to inhibition of this metabolic transport reaction which is probably of no importance to the host cells (Gale & Rodwell 1948).

Intensive study of three widely different types of drug has shown, therefore, that chemotherapeutic success is achieved in each case by inhibition of a metabolic reaction which is of vital importance to the parasite but of no particular importance to its host.

Comparative biochemistry has demonstrated many similarities between the metabolic pathways of lower organisms and animal cells. The glycolytic pathway is closely similar in muscle and some microbial cells and is mediated by the same co-enzymes. The tricarboxylic acid cycle is of importance in animal and in microbial cells; transamination is common to both; phosphorylative transfer of energy through adenosine triphosphate is common to both, and so on. We should not be surprised, therefore, when metabolite analogues of nicotinic acid, of thiamin, of riboflavin or of pyridoxal fail as chemotherapeutic remedies and cause metabolite deficiency in the host as well as in the invading organism. The anti-metabolite approach to chemotherapy demands a much fuller knowledge of the comparative biochemistry of host and pathogen than we yet possess.

Provided we admit our ignorance there is no harm in guessing at the type of metabolite analogue most likely to be chemotherapeutically useful. Indeed, until more is known of intermediary metabolism, particularly on the biosynthetic side, guesswork is essential to further progress.

In the search for metabolic reactions which are essential to pathogen but inessential to or of no importance in the host, there are two related fields which seem to merit attention, amino-acid metabolism and protein synthesis. I have already remarked on some differences in amino-acid metabolism between Gram-positive micro-organisms and their hosts; this difference might be exploited. At Hampstead we have synthesized some amino-acid analogues, but none of these have shown promise as chemotherapeutic agents (Elliott, Fuller & Harington 1948; Harris & Work unpubl.). Other workers have also explored this field without producing any useful new drug (Lichtenstein & Grossowicz 1947; Waelsch, Owades, Miller & Borek 1946; Harris & Kohn 1941; McIlwain 1941). The failure is not wholly surprising; in addition to selective toxicity a drug must also possess some degree of biological stability. The majority of natural  $\alpha$ -amino-acids undergo rapid oxidation in the animal body and their analogues probably undergo the same change. Stability can be conferred upon the amino-acid unit by combining it in peptide linkage with other amino-acids; metabolite analogues of such type offer a vast field for further exploration.

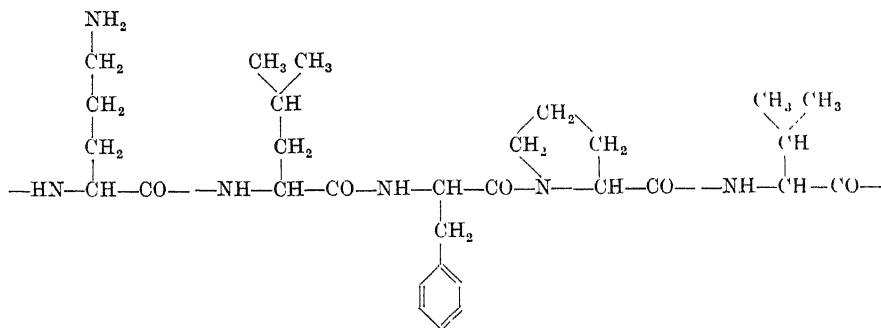
TABLE 5. ANTIBIOTIC PEPTIDES

antibiotic	source
gramicidin-D	<i>Bacillus brevis</i>
tyrocidine	<i>B. brevis</i>
gramicidin-S	<i>B. sergiev</i>
subtilin	<i>B. subtilis</i>
aerosporin	<i>B. aerosporus</i>
licheniformin	<i>B. licheniformis</i>
polymyxin	<i>B. polymyxa</i>
bacillin	<i>B. subtilis</i>
lycomarasmin	<i>Fusarium lycopersici</i>

Two known facts make the peptide field particularly attractive, the peptide nature of several natural antibiotics and the species specificity of protein synthesis.

Table 5 gives a list of some antibiotic peptides extracted from micro-organisms.

The addition of licheniformin to this list has only become possible during recent weeks. Licheniformin is a mixture of several closely related antibiotic peptides (Callow & Work unpubl.). The persistent occurrence of amino-acids of unnatural configuration in antibiotic peptides at first suggested that this might be the key to their antibiotic action. To test this hypothesis we made use of the analytical work of Consden, Gordon, Martin & Synge (1947) on gramicidin-S. The probable structure of gramicidin-S is a cyclic pentapeptide or a decapeptide with the following recurring sequence of amino-acids: ornithyl-leucyl-phenylalanyl-prolyl-valyl:



As phenylalanine is the only amino-acid of the sequence with an unnatural (D) configuration (indicated in the above formula by a side chain below the backbone of the peptide chain), we thought at first that we might achieve antibiotic action by synthesis of that part of the peptide indicated by dotted lines. Two tripeptides L-leucyl-L-phenylalanyl-L-proline and L-leucyl-D-phenylalanyl-L-proline were synthesized, but although both were slightly bacteriostatic there was no significant difference between the L-D-L and L-L-L peptides; extension of the chain to the pentapeptide stage also failed to produce antibiotic action (Harris & Work 1948), and the conclusion was therefore reached that it was the structure of the molecule as a whole rather than the configuration of one particular amino-acid which was the key to the problem (Work 1948).

The second point which lends attractiveness to the peptide field is, as already remarked, species specificity among proteins. Our search is for a metabolite analogue which will inhibit an enzymic system which is peculiar to pathogen and non-existent in its host. There is some evidence that amino-acids of peculiar pattern unknown to animal protein may be utilized by micro-organisms for protein synthesis but this is not the point to which I wish to direct attention. Practically nothing is known of the mechanism of protein synthesis, but the end-result is a molecule which is completely specific for one particular species and sometimes even specific for subgroups within that species. The mechanism for this synthesis must be equally species specific and open to selective inhibition by suitable metabolite analogues. A protein molecule contains several hundreds, or even thousands, of amino-acid residues representing about twenty different kinds of amino-acids; each kind of amino-acid is, therefore, repeated several times throughout the protein molecule to form an intricate pattern. Although the Bergmann-Niemann hypothesis of regularly



recurring amino-acid residues has not found much support in its original form, it is reasonable to suppose that in protein synthesis the living cell will, for reasons of economy, use the same amino-acid sequence in the production of a number of proteins. I wish to suggest that amino-acid sequence may to some extent govern species specificity of proteins and that a suitable peptide with a favourable amino-acid sequence for one organism may be, for this reason, highly toxic to another group of organisms.

Some evidence can be cited in favour of this view; the growth requirements of micro-organisms are not invariably satisfied by a mixture of amino-acids, vitamins and salts; one of the additional factors required by *Lactobacillus casei*, designated strepogenin, is peptide in nature and is replaceable to some extent by the synthetic peptide seryl-glycyl-glutamic acid (Woolley 1946, 1948). Since all three amino-acids are already present in the nutrient medium of *Lb. casei* it would appear that this organism requires for optimum growth a peptide containing the sequence seryl-glycyl-glutamic acid, and is unable to synthesize this sequence sufficiently readily to permit rapid growth. On the basis of the Fildes-Woods hypothesis other related peptides might be expected to be growth inhibitory. Woolley found that seryl-glycyl-aspartic acid was antibiotic for *Lb. casei* and that it possessed the characteristic biological activity of a naturally occurring antibiotic, lycomarasmin, from *Fusarium lycopersici* (Plattner & Clauson-Kaas 1945).

There are other suggestions in the literature also, that some particular sequence of amino-acid residues may be of particular importance in the metabolism of higher animals; thus, tryptic digests of lactalbumin have a higher biological value in the dog than can be accounted for by their amino-acid composition (Bolling, Block & Chow 1947); certain animal proteins seem to be a rich source of strepogenin while egg-white, gelatin and salmine are devoid of strepogenin activity (Sprince & Woolley 1945). Another small piece of evidence that peptides with one particular amino-acid sequence may be characteristic of one species derives from the observation of Dent & Schilling (1948) that a dog digests a meal of dog protein without any rise in the amino-acid content of the portal blood, whereas, after any other protein meal, there is a sudden and clearly defined increase in free amino-acids in the blood; apparently the dog is able to utilize homologous protein without breaking it down first to its constituent amino-acids, but cannot do the same with heterologous protein.

There is one final point to which I should like to draw attention in connexion with selective toxicity and antibiotic action. Some of the most potent and highly selective of all naturally occurring antibiotics are the bacterial toxins. Just because their antibiotic action is directed against animals rather than against other micro-organisms they are not generally classed as antibiotics but they nevertheless logically belong to this class of compounds. Crystalline botulinus toxin has been analyzed and found to contain fourteen amino-acids. No abnormality of amino-acid composition was found which would account for its extreme toxicity (Buehler, Bornor, Schantz & Lamanna 1946). Diphtheria toxin is another highly selective antibiotic. Growth of *Corynebacterium diphtheriae* can be greatly slowed by reduction of the iron content of the medium, as the iron content is raised, increased growth is accompanied by increased production of toxin and of porphyrin.

Beyond a critical level of iron there is a rapid fall in yield of toxin and porphyrin but growth rate continues to increase. Simultaneously with the fall in porphyrin and toxin production the cytochrome spectrum of the cell becomes strong (Pappenheimer & Hendee 1947). The suggestion has been made that diphtheria toxin is a portion of the cytochrome molecule which the cell continues to produce in media containing concentrations of iron suboptimal for cytochrome production. The biological activity of the toxin could then be regarded as due to close resemblance to the protein portion of a vitally important animal enzyme system.

If I have seemed, in the latter half of my contribution to this symposium, to allow myself a generous amount of speculation, it is because, for the present, we lack exact knowledge of intermediary metabolism, knowledge which is a prerequisite for the intelligent application of the Fildes-Woods hypothesis to chemotherapy. If my speculations in the field of peptide chemistry do something to stimulate further study of natural and synthetic peptides they will not have been entirely worthless.

#### THE DESIGN OF BACTERIAL INHIBITORS MODELLED ON GROWTH FACTORS

BY H. N. RYDON

Fildes, in 1940, put forward a plea for 'A rational approach to research in chemotherapy' in which he advocated research directed towards the preparation of modified essential metabolites which should be sufficiently closely related to the essential metabolite on which they were based as to fit the same enzyme, but sufficiently dissimilar as to be themselves devoid of essential metabolic activity. This idea was taken up widely, and fruitfully, but often without due regard to the factors making for true similarity between metabolite and inhibitor; the present paper is an appeal for such a rational approach to chemotherapy to be based on a proper appreciation of the structural factors on which such similarity must be founded.

It must be admitted that most of the metabolite analogues which have been prepared and tested as a result of the general acceptance of Fildes's idea have proved of little value as practical chemotherapeutic agents. A possible reason for this lack of success lies in the fact that the analogies between metabolite and inhibitor have been purely 'pictorial' in nature; in general, the inhibitors have simply been made to 'look like' the metabolite. In the author's opinion, Fildes's principle is unlikely to be very fruitful if we continue to confine ourselves to such naïve pictorial analogies which are both not enough and too much. Pictorial analogy is not enough because an analogue may look like a metabolite and yet lack the chemical groupings necessary for combination with the appropriate enzyme; it leads us to overemphasize the geometry and underemphasize the chemistry of the molecule. Pictorial analogy is too much, since there may be a great deal in the structure of the metabolite which is not directly concerned in its attachment to the enzyme; it leads us to overload our inhibitors with possibly unnecessary complications. In order to model inhibitors more rationally on essential metabolites we must

know two things, viz. the structure of the metabolite we are imitating and the precise way in which the metabolite combines with the bacterial enzyme to which it is related as substrate, co-enzyme or product.

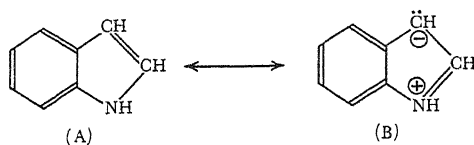
The first of these requirements should give us little trouble, since the number of known growth factors we can set out to imitate is now very large; practically, however, success or failure in the chemotherapy of a particular infection may depend on a wise choice in this respect. Regarding the second, however, there is little information available, and experiment is necessary to determine the mode of linkage of the metabolite and enzyme in each specific case; it seems likely, having regard to the ready reversibility of the type of interaction envisaged, that secondary valence forces (interaction of poles or dipoles; hydrogen bonding; van der Waals forces) rather than covalencies are likely to be involved.

It is suggested that the mode of linkage between a metabolite and its related enzyme may be determined experimentally by introducing into the metabolite molecule substituents of known electropolar properties and studying the inhibitory (or metabolic) properties of the resulting compounds. To avoid gross differences in permeability properties, the inhibitors studied should be as similar as possible and comparisons should probably be restricted to isomerides. The procedure, then, is to prepare one or more complete series of isomeric substituted metabolites and determine their activities as bacterial inhibitors; differences in inhibitory activity may reasonably be ascribed to different affinities for the bacterial enzyme and, by correlating the results with the known polar and steric properties of the substituents, it may be possible to deduce, in some detail, the nature of the combination of the metabolite and its related bacterial enzyme. If we succeed in this, it should then not be too difficult to devise other modifications of the metabolite structure leading to substances having maximal affinity for the enzyme and so maximal inhibitory activity; in this way, conscious design might supplant the empiricism which still largely governs research in chemotherapy to which we could claim to have a really rational approach.

This new method may be illustrated by some recent work of Sir Paul Fildes and the author (Fildes & Rydon 1947). A study of all the seven isomeric methyl-indoles as inhibitors of *Bact. typhosum* showed three of them (the 1-, 2- and 3-methyl indoles) to be devoid of inhibitory activity; the other four inhibited growth by interfering with the conversion of indole into tryptophan, the order of decreasing inhibitory activity being 4-Me > 6-Me > 7-Me > 5-Me. The lack of activity of all those compounds containing methyl groups in the pyrrole ring suggests that this ring is closely concerned in the combination of indole with the enzyme; any substitution here causes, probably for stereochemical reasons, a complete failure of the modified metabolite to combine with the enzyme. The alternating effect observed in the inhibitory activity of the compounds containing methyl groups in the benzene ring at once suggests that some polar mechanism is operative.

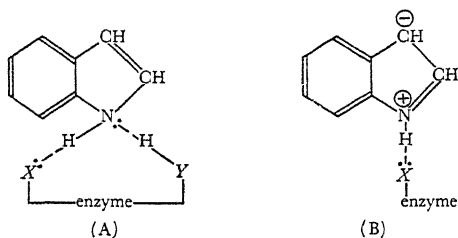
Application of the accepted principles of the electronic theory of organic chemistry (fully described in the original paper) shows that in the more inhibitory 4- and 6-methyl-indoles structure (A) will contribute more to the resonance hybrid

than it does in indole itself, whereas in the less inhibitory 5- and 7-methyl-indoles structure (B) will be more important:



It seems, therefore, that the combination of indole (and the methyl-indoles) with the bacterial enzyme concerned must be such that it is favoured by structure (A).

The two structures differ notably in their capacity for hydrogen-bonding to an enzyme since, whereas (B) can enter into only one such bond, (A) can enter simultaneously into two:

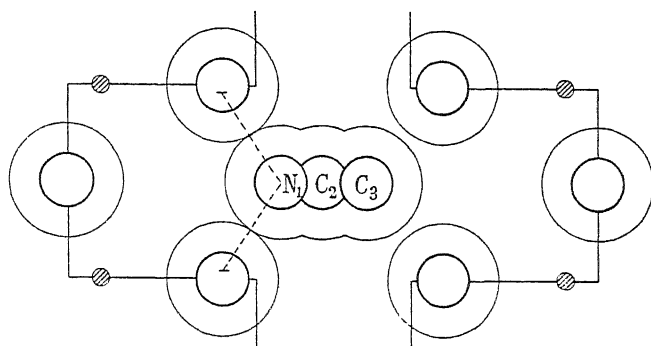


It is suggested, therefore, that the combination of indole with the enzyme involves attachment of the indole molecule, in form (A), by two hydrogen bonds to two side-chains of the enzyme molecule; such double attachment would be stronger than attachment by a single hydrogen bond, and would also result in a rigid anchoring of the indole molecule into position for further reaction.

Assuming a tetrahedral angle between the two hydrogen bonds and a hydrogen bond length of 2.9 Å, it is easy to show that the distance ( $X \leftrightarrow Y$ ) between the enzyme side-chains is 4.7 Å. Considering the uncertainties, this is remarkably close to the 5.1 Å spacing postulated in Astbury & Bell's (1941) structure for the  $\alpha$ -proteins. Scale models (figure 2) show that an indole molecule fits nicely into a fold of such a structure by double hydrogen-bonding to two side-chains; moreover, when this occurs, the atom C<sub>3</sub>, involved in the subsequent reaction with serine to form tryptophan is brought into reasonable reacting distance of the corresponding pair of side-chains in the opposite fold of the adjacent peptide chain. Such conclusions are admittedly speculative, but it is encouraging to find that they lead to so plausible a picture of the details of the combination of indole and its related bacterial enzyme.

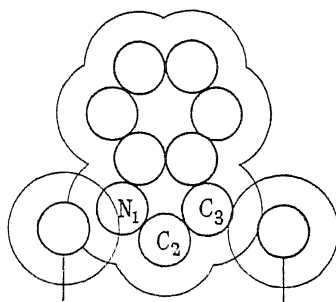
Further work on substituted indoles as inhibitors of *Bact. typhosum* is being carried out by the author and his co-workers. As a test of the hypothesis substituents of different electropolar character are being introduced into the benzene ring, while more powerfully electron-releasing groups are being introduced into the 4-position in the hope of obtaining still more inhibitory compounds.

Similar considerations can be applied to the inhibition of bacterial growth by various substituted *p*-amino-benzoic acids (Rydon 1947), but lack of time precludes their discussion now.



plan

(The broken line from  $N_1$  are the projections of  $2.9 \text{ \AA}$  hydrogen bonds. The small shaded circles indicate the positions of the side-chains projecting below the peptide back-bone, which is shown diagrammatically by the unbroken straight lines. Only  $N_1$ ,  $C_2$  and  $C_3$  of the indole molecule are shown.)



elevation

FIGURE 2. Indole and an  $\alpha$ -folded protein. (Covalent radii, thick circles, have been taken as  $0.7 \text{ \AA}$  and van de Waals radii, thin circles, as  $1.5 \text{ \AA}$ . Hydrogen atoms have been omitted.)

## SOME ANTI-MALARIAL AGENTS AS POSSIBLE GROWTH-FACTOR ANTAGONISTS

BY F. L. ROSE

Technical difficulties associated with the complex life cycle of the malaria parasite constitute a major impediment in studies of its enzyme composition. Yet such studies have been developed to a remarkable extent during recent years by the use of human, avian and monkey species of parasite, working in particular with the erythrocytic phases either in, or free from the host red cells, or in cell extracts, and it has been concluded that in many important instances, for example, in its utilization of glucose, the metabolism of the organism conforms to a pattern common among living cells. It is not surprising, therefore, that attempts to explain the mechanism of action of anti-malarial drugs should be based on the concept of the

competitive inhibition of known enzyme reactions by substances structurally related to the substrate, or product, or to enzymic prosthetic groups.

This communication is concerned mainly with the structural relationships which are apparent in varying degrees between riboflavin and certain anti-malarial substances, and the anomalies which arise when these relationships are later applied to drugs of the diguanide class.

Oerstelin (1936) was the first to suggest that mepacrine and pamaquin, by virtue of their ability to form hydro-derivatives, might act by interference with the functioning of a riboflavin nucleotide component of some enzyme essential to growth. Silverman & Evans (1944) have shown that the bacteriostatic action of mepacrine on *B. coli* is reduced by a number of substances, including riboflavin, and drew attention to the structural similarity of these compounds. In addition, they showed that cultures of the bacillus resistant to mepacrine possessed increased resistance to quinine, suggesting a common point of attack for the two drugs. Johnson & Lewin (1945) also found that the growth-inhibitory action of quinine on the same test organism, exhibited over a narrow range of concentration, was reversed by riboflavin as well as by crude cozymase. Similar results were obtained independently by Madinaveitia (1946*a*) working with *Lactobacillus casei*.

A *prima facie* case can be made out, therefore, for mepacrine and quinine as riboflavin antagonists, at any rate so far as bacterial growth is concerned. Studies on the respiration of mammalian tissue by Wright & Sabine (1944) and Haas (1944) further demonstrated that mepacrine, and, to a lesser extent, quinine, interfered with the yellow enzyme systems, and it was proposed that the drugs might act by competing with flavin nucleotides for one or more essential proteins. Hellerman, Lindsay & Bovarnick (1946) confirmed these findings but pointed out that the nucleotide-inhibitor antagonism was not at all specific for anti-malarial substances, but was readily detectable with a variety of other non-anti-malarial quinolines, and indeed with such simple compounds as aniline, at higher concentrations.

Biochemical experiments with the malaria parasite itself cannot be said to implicate the flavin enzyme systems as immediately susceptible to the anti-malarial drugs. Christophers & Fulton (1938) were the first to point out that quinine, mepacrine and pamaquin, suppressed the rapid oxygen consumption of parasitized monkey blood (*Plasmodium knowlesi*) in the presence of glucose. Similar effects were observed by Coggeshall & Maier (1941), and Silverman, Ceithmal, Taliaferro & Evans (1944). It was obviously desirable to extend these experiments to parasites separated from red blood cells, especially from nucleated red cells. Christophers & Fulton (1939) showed that haemolysates of parasitized monkey blood retained both infectiveness and some degree of respiration in the presence of glucose. More recently, Speck & Evans (1945) prepared suspensions of *P. gallinaceum* from chicken blood and investigated the action of quinine and mepacrine on some of the glycolytic enzymes thereof, and Bovarnick, Lindsay & Hellerman (1946) experimented similarly with *P. lophurae* from the red cells of parasitized duck blood. In no case did the results provide evidence for the view that mepacrine acts specifically against flavin enzymes directly associated with the parasite; indeed, some of the affected enzymes quite definitely did not belong to this chemical class. It should

be noted, however, that the presence of flavin adenine dinucleotides has indeed been reported in erythrocytes parasitized with *P. knowlesi* (Evans 1946). An additional difficulty arises from the inability to differentiate with certainty between inhibitory processes which may be either primary or secondary to the lethal action of the anti-malarial drugs, i.e. concerned directly with the parasite, or with the cells of the host. A further criticism that can be made against the interpretation of much of the data available is that in many cases the drug concentrations required to produce inhibition of enzyme activity *in vitro* bear little relationship to those that must obtain in the treatment of either experimental or clinical malaria, although Ball (1946) and his collaborators claim that in their work with *P. knowlesi* the same concentrations of quinine and mepacrine were required to suppress growth both *in vitro* and *in vivo*. Here, then, in this inconclusive state, the biological aspects of the problem must be left, and attention be given to the chemical evidence that might support the association of anti-malarial activity with riboflavin. Clearly, the final answer can only be given by a biological experiment, but the demonstration *in vitro* of anti-bacterial activity of a type antagonized by riboflavin and then of anti-malarial activity *in vivo*, with a sufficient number of distinct chemical types each bearing some structural similarity to the vitamin might nevertheless be accepted for the time being as strong circumstantial evidence in favour of the hypothesis, and if accepted by the biologist and biochemist, should encourage them to seek the final proof.

The antagonism between riboflavin (I) and the anti-malarial drugs, mepacrine (II), quinine (III) and pamaquin (IV), with respect to the growth of *Lactobacillus casei* and other bacteria has already been referred to.

Of these compounds, it can be argued that mepacrine bears the closest formal structural resemblance to the vitamin in that both contain a tricyclic system, similarly constituted in part, and each carries a side-chain attached to the ring system at equivalent positions.

The biochemical antagonism might then be due to one or more of the major differences in detail. These include the inherent chemical differences such as reducibility, between the acridine and isoalloxazine ring systems; the substitution of chlorine for the two methyl groups, which is significant in view of the observation made by Kuhn, Weygand & Möller (1943) that the 6:7-dichloro analogue (V) of riboflavin is a potent inhibitor of this substance with respect to bacterial growth; and finally, as pointed out by Madinaveitia (private communication), the opposite terminal ionic charge on the side-chain of the drug compared with that of the vitamin in its phosphorylated (mononucleotide) form.

The structural relationship between quinine, pamaquin, and riboflavin is less obvious and cannot justify argument beyond the point that both of the drugs can be regarded as containing a methoxyquinoline system in common with mepacrine.

The more recently discovered anti-malarial anilino-pyrimidines developed in these laboratories also function as riboflavin antagonists when examined with respect to *Lb. casei* (Madinaveitia 1946*a*), and, in addition, are less anti-bacterial to strains of *B. coli* which have become adapted to mepacrine, suggesting a common point of attack on this organism (Madinaveitia, private communication). These new sub-

(I)

(II)

(III)

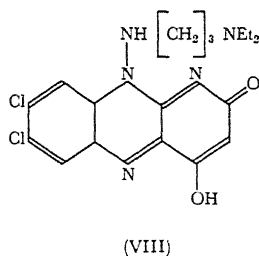
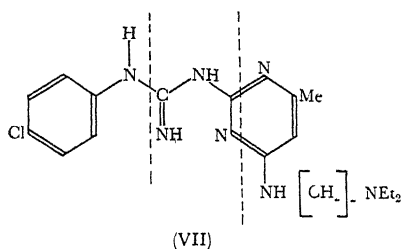
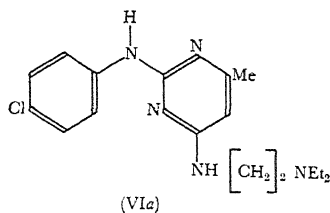
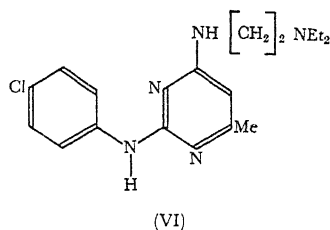
(IV)

(V)

This substance also markedly inhibits the growth of bacteria, and the inhibition is annulled by the addition of riboflavin to the culture medium. The chlorine atom is



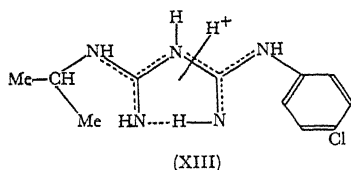
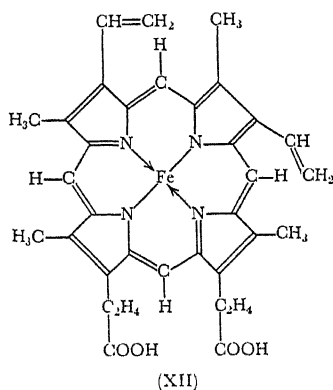
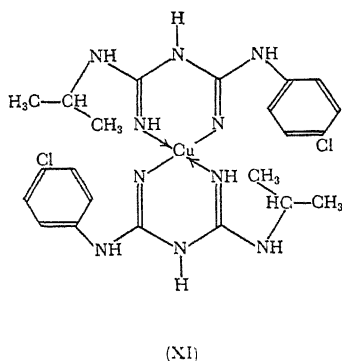
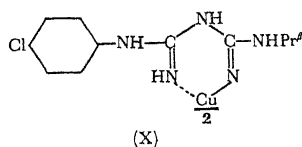
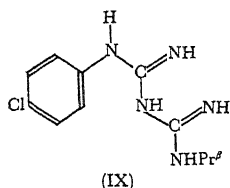
again an essential component for highest anti-malarial (and anti-bacterial) activity, and if a structural similarity to riboflavin is sought, then it must be on the basis of the correspondence of the chlorophenyl residue of the former with the xyllyl residue of the latter, and of the portion of the drug shown between the dotted lines with the pyrimidine ring of the vitamin.



Finally, an important chemical link in the hypothesis relating anti-malarial structure to the antagonism of some flavin system, is provided by the synthesis by Dr H. R. Ing (private communication; detailed results to be published shortly) of compounds such as (VIII). In these, the *isoxaloxazine* system of the growth factor has been retained, but as in (V) the 6:7-methyl groups have been replaced by 6:7-dichloro substituents and the carbohydrate group by a basic side-chain. The resultant molecule has been shown by Madinaveitia in these laboratories to be a riboflavin antagonist with respect to the growth of *Lactobacillus casei*, and by Davey to possess therapeutic activity against a *Plasmodium gallinaceum* infection in chicks.

The diguanide anti-malarial drug proguanil ('Paludrine') was derived on the basis of purely chemical reasoning made clear by comparison of its structure, when formulated in the unconventional form (IX) with mepacrine (II), and in particular with its immediate precursor (VIa). Yet Madinaveitia (1946*b*) has shown that the anti-bacterial action of this substance is not affected by added riboflavin in the culture medium, and in addition it is generally considered that quantitatively, and qualitatively, its therapeutic action in malaria is quite distinct from that of the earlier drugs. So far no explanation based on biological and biochemical research has been advanced to account for these differences. This led Curd & Rose (1947) to make proposals, based on chemical structure concepts, which they considered might merit consideration. A characteristic of (IX), and of diguanides in general, is the capacity to combine with metals, in particular with copper, to form derivatives which are undoubted co-ordination complexes. That between (IX) and copper

might be expected to have the empirical structure (X) in accord with analytical data. Molecular models showed that in the planar form (XI) this complex should possess considerable stability, and the formal resemblance of this molecule to those of the porphyrin group e.g. (XII) led to the initial prediction that proguanil might exert its biological effects through the production *in vivo* of a metal complex which would then interfere with a porphyrin-containing system associated with the malaria parasite. The most striking features of this structural similarity are considered to be the six-membered rings in which the metal atoms are chelated, the simulation of the



pyrrole rings of the porphyrin by the appropriate folding of the diguanide chain, and the coincidence of the 1:5-dimethyl substituents of the porphyrin with a pair of the corresponding methyl groups of the two *isopropyl* radicals of the drug. In the last event, the stereochemistry of the *isopropyl* groups would require the second pair of the methyl groups of the latter to be raised out of the general plane of the molecule. It may be significant that whatever chemical variations are made, either in the aromatic portion of the molecule, or along the diguanide chain, optimum anti-malarial activity where present, always requires a terminal *isopropyl* group.

Although this suggestion has been made with respect to the metal derivatives of the drug, it has since been indicated by X-ray crystallographic studies of proguanil hydrochloride in these laboratories, that the cation is essentially planar and in size conforms to the view earlier considered to be likely on theoretical grounds, and

since shown to be most probable by the physico-chemical studies of Mr J. C. Gage (to be published), that it exists as a pseudotriazole (XIII) involving a hydrogen bond.

Clearly such a structure, although not a metal complex, still bears a shape relationship with the porphyrin type (e.g. upper half of XII), and may thus interfere in biological reactions involving a porphyrin-containing system without itself being associated with a metal ion.

So far, the small amount of biochemical work carried out in these laboratories to check this suggestion has not afforded any support for it. Dr Madinaveitia has not discerned any interference with a root peroxidase system, nor does proguanil specifically inhibit the growth of *H. influenzae*, which organism requires a source of haematin in the culture medium. Some inhibition does occur, but this was shown to be due to the formation of a sparingly soluble salt between the drug and haematin, a property also exhibited by quite a range of other cationic substances.

It is known, however, that the heavy metal catalysts of the Warburg-Keilin cytochrome system are almost certainly involved in the respiratory mechanism of the malaria parasite, and the pigment of *Plasmodium knowlesi* and *P. gallinaceum* has been confirmed (Rimington, Fulton & Sheinman 1947) as haematin.

If the possible significance of the porphyrins in relation to the anti-malarial activity of the diguanide derivatives could eventually be supported by experimental evidence, then since the latter were in turn closely derived from the earlier 'riboflavin-simulating' type, it is tempting to suggest that the flavin systems also bear some structural relationship to those of the porphyrin group. The close association of the two types in enzymic dehydrogenase reactions might then be viewed in a light, which so far as the author is aware, would be novel. At this stage of our knowledge, further speculation would be idle, but on a purely chemical basis it might be stimulating to conclude by pointing out one potential point of resemblance, namely, that the riboflavin molecule contains a system in the 4-hydroxyisoalloxazine residue potentially capable of chelating a metal atom (compare Albert & Magrath 1947).

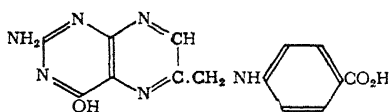
#### GENERAL DISCUSSION

J. WALKER

The idea of a drug interfering with the utilization of an essential metabolite is older than the subject of chemotherapy itself, and probably celebrated its fiftieth birthday about a month ago. In 1898 Ehrlich, in a letter to his cousin Carl Weigert, indicated clearly the conception which has attracted so much attention in recent years. In his example Ehrlich pictured, for the sake of argument, an aldehyde group protruding from a cell having the particular function of fixing a specific nutritional factor for that cell. He then pictured a harmless compound, circulating in the blood, having the property of combining with this aldehyde group and so rendering the latter unavailable for its normal role. He goes on to say that the cell in this specific way must go hungry and can only survive if it can throw out another side-chain carrying an aldehyde group (Heymann 1928). In modern nomenclature,

a drug may occupy the site on an enzyme normally utilized by an essential metabolite and, as Fildes pointed out in 1940, if the drug is similar to the metabolite in general structure, yet not sufficiently similar to have the same biological action, it has a good chance of interfering with that metabolite by competing with it for its enzyme. I do not know if any writers on this subject go beyond this and enquire what happens to the drug, but it was suggested by Andrewes, King & Walker (1946) in discussing the action of *p*-sulphonamidobenzamidine and related compounds in experimental typhus that these drugs were incorporated into functionally useless structures by the organism with consequent squandering of its synthetic resources. Such a view may sound heretical to many enzymologists but we have a good analogy in the work described from the Lilly Research Laboratories (1945), where unnatural phenylacetic acids were presented to *Penicillium notatum*, either as ethanolamides or as amides with DL-valine, with consequent production of analogues of penicillin G in which the phenylacetyl group of the latter has been replaced by the corresponding acyl radicals from the unnatural phenylacetic acids; incidentally, it would be a great convenience in the nomenclature of the penicillins if a trivial name could be adopted for the amine of which penicillin G is the phenylacetyl derivative. Similarly, with the knowledge that *p*-aminobenzoic acid turns up in the folic acid molecule, it seems likely that sulphonamides may be built up into functionally useless structures and that they do not merely compete with *p*-aminobenzoic acid for a site on the enzyme utilizing *p*-aminobenzoic acid in the fashion of a game of musical chairs.

Forrest & Walker (1948) have suggested that the three carbon atoms of pterotic acid indicated in heavy type may be derived from a triose, and the suggestion by O'Meara, McNally & Nelson (1947) that the strongly-reducing non-sulphydryl substance found in bacterial cultures during the logarithmic phase of growth might be reductone,  $\text{CHO} \cdot \text{C}(\text{OH}) : \text{CH}(\text{OH})$ , seemed to us to be significant in that connexion.



O'Meara and his co-workers suggested that *p*-aminobenzoic acid acts as a stabilizing agent for reductone in bacterial metabolism, enabling the cell to store reductone and to utilize it as required, presumably after hydrolysis of the reductone—*p*-aminobenzoic acid condensation product to free reductone. They also suggested that sulphonamides interfere with bacterial growth by combining with reductone and preventing it from becoming available for the use of the cell. Another possibility which presents itself is that, in normal bacterial metabolism, *p*-aminobenzoic acid and reductone are first linked together and then incorporated into pterotic acid and the other factors of this group, such as pteroylglutamic acid, whereas, in sulphonamide bacteriostasis, the drug combines with reductone, and the condensation product is incorporated into a biologically inert analogue of pterotic acid.

Using the condensation product of reductone with methyl *p*-aminobenzoate we have obtained pterotic acid on condensation with 2:4:5-triamino-6-hydroxypyrimidine, and similar condensations with a variety of sulphonamides have

provided corresponding compounds in which the carboxyl group of pterioic acid has been replaced by the appropriate substituted sulphonamide groups. Our colleague, Miss Horton, has examined two of these, namely those with an unsubstituted sulphonamide group and a methylsulphonyl group respectively in place of the carboxyl group of pterioic acid, and both act as antagonists of pterioic acid and of pteroylglutamic acid in cultures of a strain of *Streptococcus lactis* requiring one or other of the latter two substances. We have now extended this study to include the analogue of pteroylglutamic acid in which the  $\text{—CO.NH—}$  linkage has been replaced by the  $\text{—SO}_2\text{.NH—}$  group, but no biological results are yet available.

# A. ALBERT

Although oxine (8-hydroxyquinoline) has been used as an anti-bacterial since 1895, its mode of action remained obscure until recently. Hata's (1932) suggestion, that oxine combines in the one molecule all the virtues of phenol and quinoline, could hardly be classed as a scientific explanation.

In some work which I carried out in Australia in collaboration with Rubbo, Goldacre & Balfour (1947), it became apparent that oxine was injuring bacteria by combining with trace-metals present in either the medium or the organisms. Oxine has been used for some years by analysts to de-ionize solutions of metallic salts by the type of combination (known as chelation) which is shown in figure 3. The metallic ions, when bound in these complexes, lose their inorganic nature and begin to resemble organic substances, e.g. the complexes become soluble in chloroform.

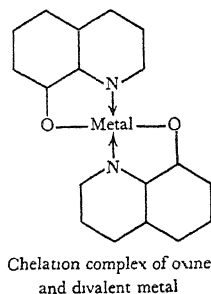
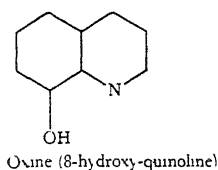


FIGURE 3.

The biologically interesting metals chelated by oxine at pH 7 were found (Albert & Gledhill 1947) to be copper, cobalt, lead, zinc, cadmium and manganese (all strongly) and iron (less strongly), whereas magnesium and calcium were hardly chelated at all except in alkaline solutions. This survey was extended to 45 deriva-

tives of oxine (Albert & Magrath 1947) and it was found (Albert *et al.* 1947) that there is a strong correlation between chelation and anti-bacterial activity. For example, the six isomerides of oxine do not chelate and are not anti-bacterial, whereas oxine which chelates strongly is anti-bacterial against Gram-positive bacteria\* at a dilution of  $M/100,000$ . Again, neither the *O*-methyl-ether of oxine nor the *N*-methyl (quaternary) derivative can chelate and neither is anti-bacterial.

Assuming that oxine acts by withdrawing an essential metal from bacteria, it was hoped to discover the nature of this substance by seeing which metal could prevent oxine from acting. The result was more clear cut than we had expected, because only cobalt was able to prevent the action of oxine on Gram-positive organisms whereas this metal had almost no effect on Gram-negative rods; the latter, however, were protected from the action of oxine by either copper or zinc. One possible interpretation of these results is that cobalt may have some important function in Gram-positive organisms.

So far, only bacteriostatic tests have been discussed, but bactericidal tests gave results which were much more clear cut. A dilute solution ( $M/50,000$ ) of oxine is completely lethal in about 20 min. to a dense culture of *Staphylococcus aureus*, but a more concentrated solution ( $M/3000$ ) is not completely lethal in 24 hr. I need not emphasize that it is unusual for a disinfectant to become weaker as its strength is increased! At this stage, the following hypothesis was formulated: 'Oxine may act in dilute solution by removing a protecting metal (e.g. cobalt), thus exposing the bacteria to the action of a second metal which catalyzes the oxidation of the chemical group which the former metal protected. Stronger solutions of oxine may protect the organism by also removing the destructive metal.'

The following results, which Professor Rubbo has recently obtained, suggest that the injurious metal is (ferrous) iron and that the organisms actually die of iron poisoning. When the usual broth medium was repeatedly shaken out with oxine and chloroform (12 times each, in alternation), it apparently became highly deficient in iron. Staphylococci still grew in it, but neither strong nor weak solutions of oxine could kill them. When, however, a trace of ferrous sulphate was added they at once became vulnerable to weaker solutions of oxine, although ferrous sulphate on its own was harmless. Again, staphylococci would live for some time in triple glass-distilled water, and in this liquid they were not vulnerable to oxine. However, they became vulnerable as soon as a trace of ferrous salt was added.

Iron-poisoning has been discussed lately in another branch of biochemistry. Racker & Krimsky (1947) have suggested that encephalomyelitis virus acts by transporting ferrous ions across the blood-brain barrier and they have shown (Racker & Krimsky 1948) that iron strongly and specifically inhibits the oxidation of phosphoglyceraldehyde in normal glycolysis. We should be very interested to hear of other specific examples of poisoning by iron.

If the above case for metal-metal antagonism can be further substantiated, it will establish in chemotherapy a phenomenon that is already well known to workers in agriculture and animal husbandry.

\* Gram-negative organisms are not so sensitive and many require  $M/2000$ .

M. STACEY

In considering the method of approach to an understanding of the mode of action of anti-bacterial agents it is a striking fact that members of the Gram-positive group are in general more readily affected than those in the Gram-negative class. It is felt that a knowledge of the chemistry of the magnesium ribonucleo-protein is a prerequisite to further research. This nucleoprotein belongs to the class of auto-synthetic molecules and contains enzymes or factors responsible for biomolecular synthesis of macromolecules including capsular polysaccharides.

Bacterial growth is essentially the uninterrupted building up in an orderly fashion of big molecules, i.e. it involves very largely 'biomolecular polymerization'. Where proteins are concerned it involves also the specific assimilation of amino-acids. There is a delicately poised balance between the build-up or polymerization systems and the breakdown or lytic systems. Any chemical substance which can interfere with any one of the build-up systems may affect bacterial growth and disturb at least some function, e.g. the virulence of the invading agent. This is particularly so with capsular polysaccharide synthesis. At least one strain of leuconostoc species needs P.A.B. for polysaccharide synthesis and it is tempting to speculate that the pneumococcus may need P.A.B. for capsule production and that the sulphonamides act by inhibiting the capsule formation by exclusion of P.A.B.

It has been shown that a minute dose of penicillin will cause remarkable snake-like forms of *Cl. welchii*. It has been demonstrated that the ratio and amounts of the two forms of nucleic acid in these organisms are unaffected by the drug but that proteins are altered—possibly to forms having a more fibrous structure. It appears that in such an unnatural system involving the biomolecular polymerization of amino-acids to proteins, a 'chain breaker' is lacking. It might be that if as Gale has shown, penicillin can exclude the uptake of glutamic acid, then this amino-acid itself may be a chain breaker in a certain specific sequence of amino-acids.

A further point of note is the fact that the magnesium ribonucleate of the Gram complex contains magnesium in a co-ordinated state whereas Gram-negatives contain only ionic magnesium.

The mechanism of magnesium intake must now be studied because its partial exclusion during growth also causes in *Cl. welchii* the generation of snake-like forms which are unsegmented.

Here again it is clear that magnesium plays a vital role in biomolecular polymerization.

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# Fatigue and neuromuscular block in mammalian skeletal muscle

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Records have been taken of the tetanic tensions of the decerebrate cat's soleus and tibialis anterior muscle, while these were excited maximally by direct electrical stimulation, or through the nerve at frequencies up to 250/sec. and for some 20 sec. The tension-time curves with both methods of stimulation were almost identical for any given frequency.

Assessment of the neuromuscular block as fatigue progresses was made from observation of the tension changes which followed a sudden switch from direct to indirect excitation or vice versa.

At frequencies above 40/sec., block can be demonstrated before there is any fall in tension.

The block which develops is not absolute; fibres to which transmission is failing respond to some, but not to all nerve impulses. This enables block to be measured in terms of the average response frequency of the fibres.

The response frequency of the muscle fibres during a tetanus depends only on the total number of stimuli which the nerve has received. The relation is of the form

$$\text{response frequency} = \frac{a}{(\text{total stimuli})^b},$$

where  $a$  and  $b$  are constants independent of frequency and duration of nerve stimulation. These constants are such that the average response frequency of the muscle fibres has fallen to 25/sec. after the nerve has received about 1000 stimuli at a frequency of 100/sec.

Fibres which are rested by block can, when they do respond, develop up to three times the tension-time of unfatigued fibres.

The development of neuromuscular block is not responsible for the fall of tension which occurs as the system fatigues.

## INTRODUCTION

The tetanic tension developed by a muscle, excited through the motor nerve with repeated maximal stimuli, declines as stimulation continues. Provided that the stimulation is not unduly prolonged or of too high a frequency, the decline in tension is reversible, and repetition of the same stimulation produces the same tension changes. Stimulation of a mammalian nerve-muscle preparation for some 2 hr. at frequencies as low as 1 in 10 sec. admittedly produces a series of twitches whose peak tensions gradually decline, but this fall of tension is not reversible within the limits of an acute experiment. In this paper we shall restrict the meaning of the term 'fatigue'; it will be taken to imply a decline in tetanic tension which is reversed by allowing the muscle to rest for about 10 min.

Part of the decline in tension which follows prolonged stimulation of the motor nerve has often been attributed to failure of transmission from nerve to muscle, and the existence of neuromuscular block has been demonstrated often enough (Lucas 1917; Davis & Davis 1932; Feng 1937; Rosenblueth & Morison 1937). Despite this, very little is known of the extent to which failure of transmission may contribute to the fall of tension in a fatigued muscle, or of the site of neuromuscular block. Davis & Davis (1932) concluded that in the cat's soleus and

gastrocnemius muscles 'at most only 14.5 % of the fibres could have been idle during tetanic stimulation' (at 30/sec.), although there was a much greater proportional fall in tension. On the other hand, Rosenblueth & Morison (1937), who also worked with the muscles of the cat's leg, state that it is 'possible to account for the fall of tension during a tetanus in terms of idle muscle fibres, although the nerve volleys remain maximal throughout'.

The purpose of the work we describe was to obtain some quantitative estimate of the importance of neuromuscular block in reducing the tetanic tension of a fatigued muscle. We are not concerned in this paper with the site at which such block occurs. We have confirmed the statement that failure of transmission from nerve to muscle can occur in mammalian preparations under conditions of stimulation which do not cause irreversible changes in the system, but found to our surprise that the development of block contributes little or nothing to the decline in tetanic tension.

#### METHODS

The nerve-muscle preparations were all from the hind legs of cats decerebrated under ether. The muscles tested were the soleus and tibialis anterior, and details of the exposure of nerve and muscles will be found elsewhere (Brown 1938; Eccles & O'Connor 1939). The sciatic trunk was tied central to the point of application of the stimulating electrodes, and all branches except those to the muscle under test were cut. In order to ensure that the current supplied for direct stimulation was not short circuited, the muscles were dissected free of the surrounding tissues so far as was possible. For the tibialis muscle, insulation was maintained by enclosing the belly of the muscle in a rubber finger-stall wetted with liquid paraffin; finally, the skin was sewn together over the rubber sheath, with no more than the tendon projecting. It was found convenient to prepare the soleus so that the whole muscle lay in a bath of paraffin; the walls of the bath were made by skin flaps suspended so that the muscle lay in a sloping gutter with the tendon emerging from the upper end. The limb was fixed by drills in the tibia and femur so that the muscle under examination lay approximately in the horizontal plane. The temperatures of the muscle and of the cat were measured throughout the experiment by means of thermocouples.

Tension records were usually made by attaching the tendon to a condenser-myograph. This instrument allows the muscle to pull against a spring which holds one plate of an electrical condenser in position. An applied tension increases the distance between the condenser plates and the electrical capacity falls. The tension is measured by use of an electrical system which records changes of condenser capacity as deflexion of a cathode-ray beam (Buchthal 1942). This instrument was designed for distortionless recording of single twitches, and we have only used it for recording tetanic tension in these experiments as a matter of convenience. All of the work which we describe could, no doubt, have been performed equally well with a smoked drum and spring myograph; in fact, in a number of experiments described towards the end of this paper we used this method.

Stimuli were applied to the nerve through shielded silver electrodes which were connected to the 'floating' secondary of a transformer so that the proximal elec-

trode was the anode. The stimuli were electronically controlled rectangular waves of voltage, each with a duration of  $70\ \mu\text{sec}$ . The minimal voltage required to give a maximal twitch was determined, and then the stimulator was set to provide stimuli of ten times this strength (2 to 4 V).

The direct stimuli were applied between the tendon of the muscle and the drill in the femur. Electrical contact with the tendon was made through silver wire attached to a saline-soaked pad of cotton-wool. The stimuli were again controlled electronically and were 'floating' rectangular waves of current, each lasting  $300\ \mu\text{sec}$ .

It is most important that both types of stimulus should be provided from floating circuits, since any electrical continuity between the systems for direct and indirect stimulation leads to anodal block of the nerve which persists after a period of direct stimulation. Even with the mutually insulated stimulators we have used, a limit is set to the current which can be used for direct stimulation, since too high a current still produces transient nerve block. As fatigue progresses the excitability of the muscle fibre to direct stimulation decreases rapidly. This implies that maintained maximal stimulation requires the greatest stimulus strength possible. In practice we have always adjusted the current of the direct stimulus so that it was just below that which produced subsequent nerve block in the following test. The test current was applied continuously to the muscle for a time  $T$  sec. where  $T = Nd$ , and the subsequent response of the muscle to maximal nerve volleys at 2 or 3/sec. was observed;  $N$  = the greatest number of direct stimuli to be used in any test tetanus, and  $d$  = the duration of each stimulus. The 'safe' direct currents found by this method were usually about 20 to 40 mA. Such stimuli applied to the muscle stimulate the whole neuromuscular system. Although they occasionally became inadequate for maximal excitation of an extremely fatigued system, the stimuli were always adequate for direct maximal excitation of the rested but fully curarized muscle. 'Instantaneous' switching from stimulation of the nerve to direct excitation of the muscle was carried out by telephone relays. Because the operation of these takes some 10 msec. there is occasionally a slight irregularity in the tension records at the moment of switching. The total period of stimulation was usually about 20 sec.; 10 min. complete rest was allowed after each tetanus.

## RESULTS

Nearly all the experiments were concerned with the changes in tetanic tension which occur when maximal repeated stimulation of the nerve is 'instantaneously' exchanged for maximal repeated stimulation of the muscle, or vice versa. The general form of the results was the same for both soleus and tibialis preparations; quantitative differences were observed and are referred to when they are relevant.

### *Tests for the presence of neuromuscular block*

#### *The tetanic response to uninterrupted direct or nerve stimulation*

In a preparation which has been dissected without overt damage to any branches of its motor nerve, the peak tension developed by the single twitch is the same (to within 5 %) whether the muscle is stimulated through its nerve or by a stimulus

applied directly, between the tendon of the muscle and its bony origin. In the same way, the tension developed in the first 0.5 sec. of a tetanus is independent of the type of maximal stimulus employed. The subsequent course of the tetanic tension-time curve depends upon the frequency of stimulation used; in its general form the curve is the same whether the muscle receives stimuli through its nerve or directly. At frequencies up to about 100/sec. the initial sharp rise of tension is followed by a slow increase which reaches a maximum in 5 to 10 sec.; afterwards tension declines. At frequencies of stimulation above 150/sec. the initial rise of tension is followed immediately by a continuous decline. At intermediate frequencies (100 to 150/sec.) the tension remains practically constant for some 5 sec. before declining (figure 1).

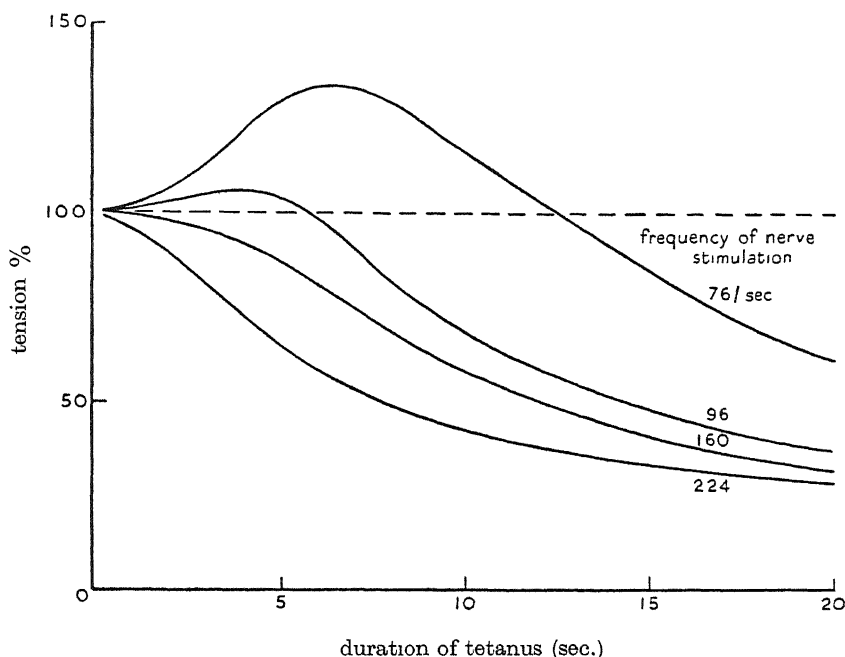


FIGURE 1. Time course of tetanic tension of soleus for various frequencies of nerve stimulation.

The decline of tension when excitation is through the nerve is often slightly more rapid than that which occurs during direct stimulation at the same frequency, but this is not always so. In some experiments, indeed, we have found that the tetanic tension declines *less* rapidly with stimulation of the nerve. The difference between the two curves is very variable, but the maximum difference rarely exceeds 15 %.

At first sight a deficiency of say 10 % in the tension evoked by nerve stimulation, compared with the tension from direct stimulation, might be taken to imply a transmission block involving 10 % of the neuromuscular junctions; an equality of the tensions would then imply absence of block, and the instances where the tension from nerve stimulation exceeded the tension from direct stimulation could only be explained by ultimate submaximality of the direct stimulus. Further analysis showed, however, that these simple explanations were untenable.

*Tests for neuromuscular block during the course of a tetanus  
produced by stimulation of the nerve*

In these experiments a tetanus was produced by maximal stimulation of the nerve at various frequencies. At intervals, during the course of this tetanus, the stimulus was switched from nerve to muscle so that for test periods of 0.75 sec. the system was excited at the same frequency by a stimulus adequate to produce direct excitation of all the muscle fibres and, of course, of all the intramuscular nerves. Short test periods of this type are not sufficient to disturb the general course of the tension-time curve produced by stimulation of the nerve alone.

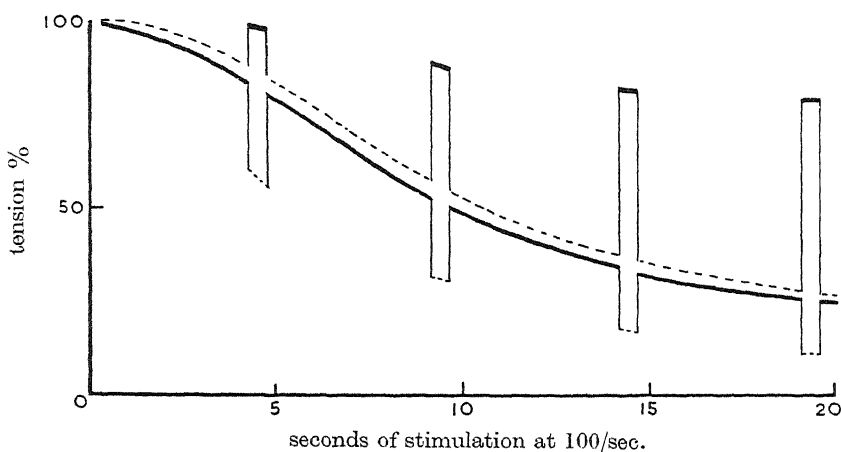


FIGURE 2. Changes in tension of tibialis when nerve stimulation is exchanged for direct stimulation at the same frequency. Upper curve, nerve stimulation interrupted by direct stimulation. Lower curve, direct stimulation of the same muscle interrupted by nerve stimulation. — Tension due to direct stimulation; - - - tension due to nerve stimulation.

A sudden switch to direct stimulation after a period of nerve stimulation produces an immediate and maintained increase in tension (figure 2). This occurs even in a muscle whose tension is better maintained by nerve stimulation than it is by direct excitation. To illustrate this point we have purposely chosen the records presented in figure 2 in which the curve for continuous nerve stimulation falls slightly above that for direct stimulation, although they do not represent the average result. The tension produced by the switch-over from nerve to muscle stimulation is always greater than the tension produced by direct stimulation for a similar length of time. This apparent anomaly can only mean that muscle fibres, failing to respond to the nerve volleys, were brought into action by the direct stimulus. Differences (or the occasional absence of difference) between the fatigue curves for direct and for nerve stimulation are consequently no index of the presence or absence of neuromuscular block. We therefore conclude that failure of neuromuscular transmission occurs even in preparations in which the tension declines as rapidly with direct stimulation as it does from excitation through the nerve.

The increase in tension on switching from nerve to direct stimulation, when expressed as a fraction of the tension due to direct stimulation, bears an ap-

proximately linear relation to the total number of stimuli applied to the nerve (figure 3). This relationship appears true for frequencies from 50 to 200/sec. The useful duration of the tetanus is limited by two factors; a very prolonged tetanus results in fatigue which is in part irreversible, or reversible only after an inconveniently long interval, and severe fatigue reduces the direct electrical excitability of the muscle fibres to an extent which makes maximal excitation very difficult.

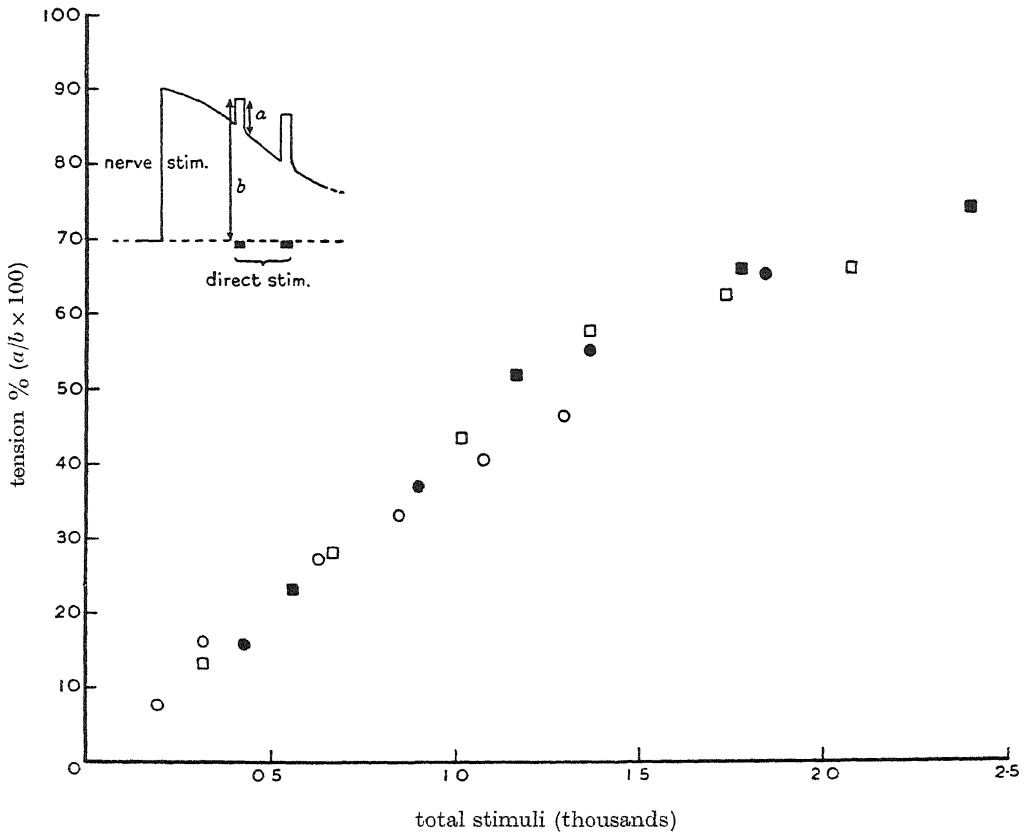


FIGURE 3. Increase of tension of tibialis produced by switching from nerve stimulation to direct stimulation at the same frequency, expressed as percentage of tension due to direct stimulation (see small figure) and plotted against total stimuli for various frequencies. Stimulus frequencies/sec. used:  $\circ$  = 46,  $\square$  = 74,  $\bullet$  = 100,  $\blacksquare$  = 130.

#### *The quantitative estimation of neuromuscular block*

The experiments which we have just described show beyond question that neuromuscular block can occur with repeated stimulation of the motor nerve. They were originally designed to provide a quantitative measure of neuromuscular block, since we had assumed that, once block had occurred, it would persist, and that the increase in tension, when the muscle was directly excited after a period of nerve stimulation, would be due to the contraction of fibres previously completely idle. The change in tension on switching the stimulus from nerve to muscle, or vice versa would then have provided a measure of the number of idle muscle

fibres. The results of these preliminary experiments have shown, however, that the assumption of persisting block is not permissible, since it offers no explanation at all of the fact that the tetanic tension curve due to nerve stimulation may be the same as or, on occasion, rise above that produced by direct stimulation at the same frequency. An alternative hypothesis was that the neuromuscular block occurring in fatigue was not 'all or nothing', in the sense that it could be assessed in terms of the number of fibres to which nerve transmission had entirely failed, but that it was graded, muscle fibres responding at first to all nerve impulses, then perhaps to nine out of ten, and so on until they might respond only occasionally. This hypothesis can explain the close concurrence of the fatigue curves of the muscle excited through its nerve and of the muscle directly stimulated, if we assume that fibres which were rested by partial neuromuscular block could, when they did contract, make a much greater contribution (tension  $\times$  time) to the total tension than the contribution possible had they responded to every stimulus.

We therefore made further experiments to test the validity of these two hypotheses.

#### *Evidence from action potentials*

If our assumption about the character of neuromuscular block was true, then indicative changes should be observed in the action potential of a muscle fatigued through its nerve.

We expected that the action potentials led from small groups of fibres within the muscle bulk would become extremely variable immediately transmission block

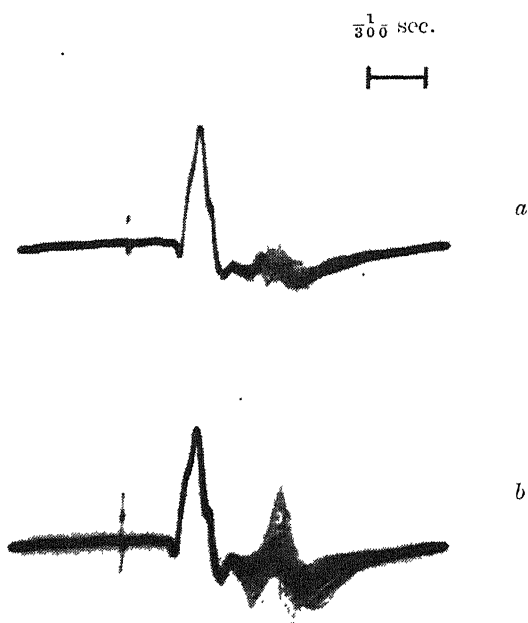


FIGURE 4. Soleus preparation. Complex action potentials recorded with microelectrodes 1 mm. apart in the muscle belly, showing the responses of two predominant fibre groups of very different latencies. Each record shows 30 to 50 superimposed traces of successive responses. (a) After 1870, and (b) after 2100 stimuli to the nerve.



set in and before there was a large decline in total tetanic tension, since units within the microelectrode span might be expected to respond to some but not all nerve volleys. Figure 4 shows this to be true for those groups of muscle fibres responding with the greater latency. It shows also that once the action potential has begun to fluctuate with successive stimuli, occasional potentials may be greater in magnitude than any recorded just before alternation became apparent.

*Estimation of average response frequency of muscle fibres*

The electrical records lend support to the view that during prolonged stimulation of the motor nerves, the muscle fibres do not follow the rhythm of nerve impulses but respond at a lower average frequency. Whether or not there are a few fibres which become entirely inexcitable to incident nerve impulses, we have no means of telling, but in either case we can justifiably refer to the average response rate of the fibres, which must fall below the frequency of nerve volleys as fatigue progresses. We therefore regard the changes in tension which have been described, when the stimulus was switched from nerve to muscle or vice versa, as being caused by sudden increases or decreases of the average response frequency of the muscle fibres.

The purpose of the experiments described below was to obtain an estimate of this average frequency of response. The effective neuromuscular block would then be measured as

$$1 - \frac{\text{average response frequency of muscle fibres}}{\text{frequency of maximal nerve volleys}}.$$

The tension changes which follow a switch of the stimulation from nerve to muscle cannot conveniently be used as a direct measure of muscular response frequencies, since the curve relating frequency of direct maximal stimulation and tetanic tension, shifts continuously as the muscle fibres fatigue (figure 5).

It became clear that a procedure calculated to take into account the many variables concerned in the progress of fatigue was to imitate the course of tetanic tension due to stimulation of the nerve at a fixed rate, by direct stimulation at a frequency which was measured and under the control of the observer.

The tetanic tension of the preparation was recorded on a smoked drum so that at any one moment the whole course of the tension record could be seen. The nerve was stimulated at the fixed frequency chosen for testing, and, at intervals during the course of the tetanus, maximal direct stimulation was substituted for the nerve volleys. The frequency of direct stimulation was not the same as that used for excitation of the nerve, but was under the control of an observer, who attempted to adjust the frequency so that at the switch-over no change in tetanic tension occurred, and the general progress of the tension-time curve was unaltered. The frequency of direct stimulation used by the observer in his attempts to balance the muscle tension was continuously recorded. Figure 6 shows the type of record obtained. The dotted line shows a superimposed record of tension due to uninterrupted stimulation of the nerve at the same frequency; imperfect 'balancing' of the two tensions appears to disturb the progress of the record very little. The

frequency of direct stimulus necessary to 'balance' the tension due to nerve stimulation at any point, is taken as a direct measure of the average frequency with which the muscle fibres were responding to the nerve volleys.

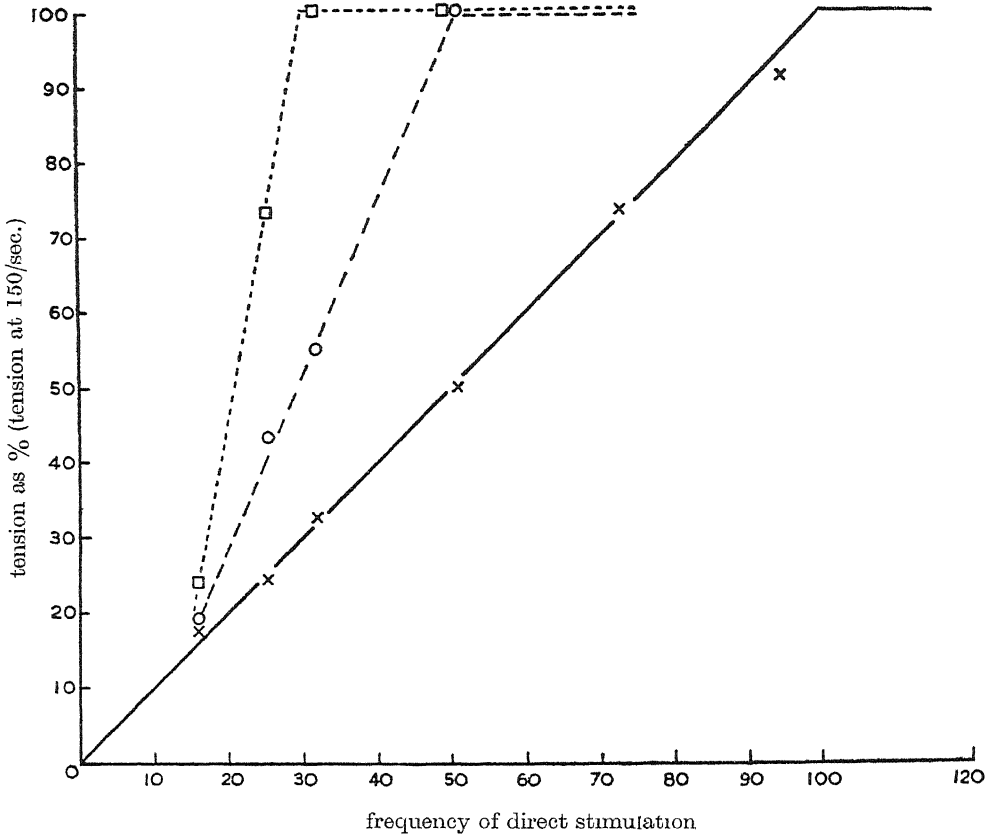


FIGURE 5. Relation between frequency of direct stimulation and tetanic tension in rested and fatigued tibialis muscle.  $\times$ — $\times$  unfatigued;  $\circ$ — $\circ$  after 15.5 sec. tetanus;  $\square$ — $\square$  after 26.5 sec. tetanus.

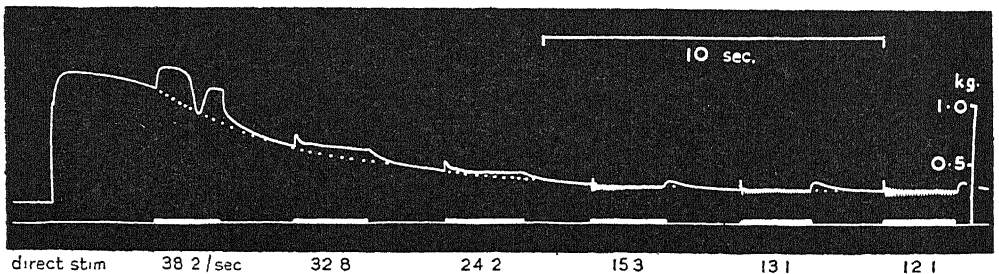


FIGURE 6. Tension of tibialis when stimulation through the nerve is interrupted by direct stimulation with frequency under control. The frequencies of direct stimulation shown below indicate the frequency at which the best match was obtained. The interrupted line shows the course of tension uninterrupted by direct stimuli. For further details see text.

In these experiments we were matching the tetanic tension due to a synchronous excitation of muscle fibres (direct stimulation) against the tension due to the asynchronous contraction of the same muscle fibres at the same average frequency. We have been able to show in a limited way that tetanic tension is independent of the synchrony of response of the muscle fibres and dependent only upon their average response rate. Figure 7 shows the tensions derived from a tibialis preparation in which a part of the muscle was excited by electrodes placed on one branch of the motor nerve, while the remainder of the muscle could be excited by electrodes placed on another branch of the nerve trunk. We excited both nerve branches at the same frequency, but alternately excited them simultaneously or with a phase difference of  $180^\circ$ . The record shows that the mean tetanic tension is insensitive to these variations and depends only on average response frequency.

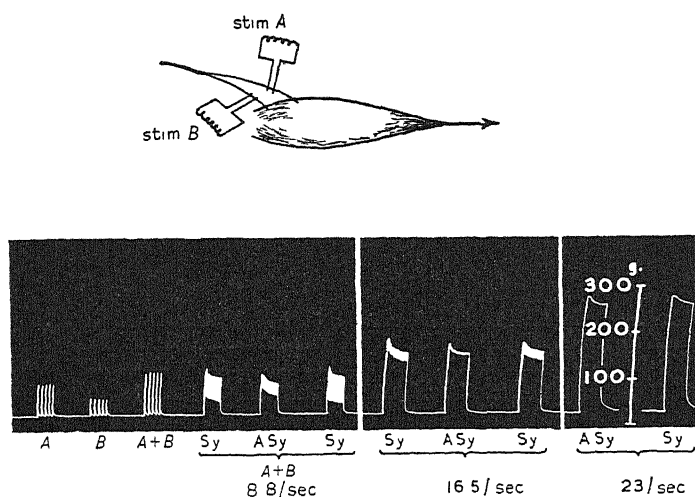


FIGURE 7. Comparison of tensions due to synchronous and asynchronous stimuli to two parts of tibialis at various frequencies. The asynchronous stimuli *A* and *B* are  $180^\circ$  out of phase.

Figures 8 and 9 show typical estimates of response frequency determined by the method we have described. The arrows attached to some of the points plotted show that the match was imperfect and indicate the direction in which true balance must lie.

The results of experiments carried out with muscles from eight cats (4 soleus and 4 tibialis) seem to justify the following generalizations:

- (1) Average response frequencies of fibres from the same muscle in different cats are remarkably alike for the same conditions.
- (2) The response frequency is determined solely by the total number of volleys which have passed down the nerve.
- (3) The behaviour of tibialis and soleus is similar. Block appears to develop rather less rapidly in soleus.
- (4) The relation between muscular response frequency  $F$  and total nerve volleys  $N$  is of the form:

$$F = \frac{a}{N^b},$$

where  $a$  and  $b$  are constants independent of stimulus frequency.

The results show a linear relationship between  $\log F$  and  $\log N$  over a frequency range from about 40 to 250/sec., and for durations of tetanus providing between 500 to 4000 stimuli. There is a general tendency for the measured frequency

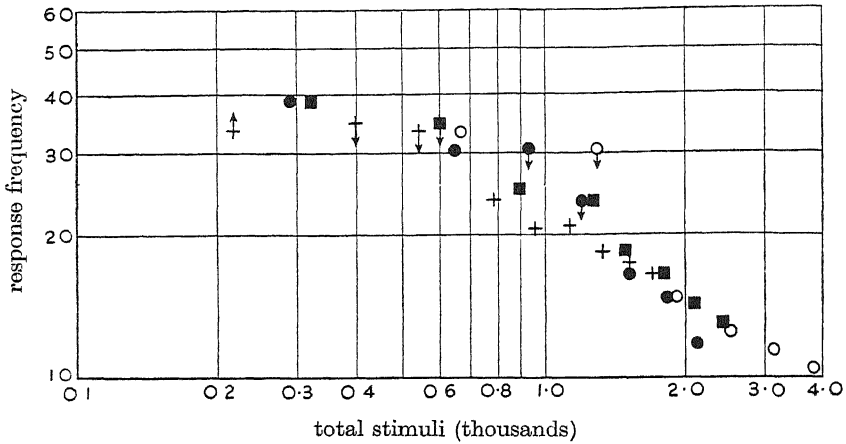


FIGURE 8. Average response frequency of muscle fibres of tibialis plotted against total stimuli to nerve. Frequencies of nerve stimulation/sec.: ● = 76, ○ = 155, ■ = 76, + = 44.

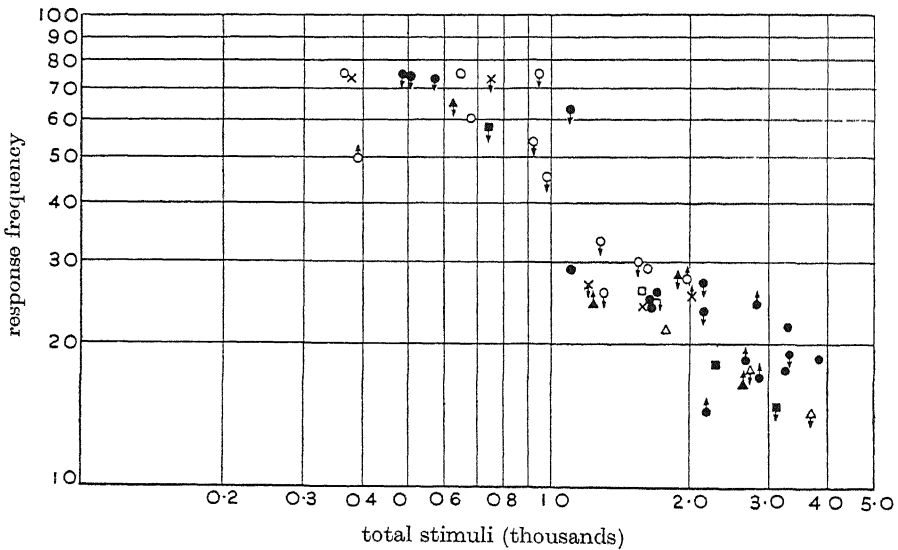


FIGURE 9. Average response frequency of muscle fibres of soleus plotted against total stimuli. Frequencies of stimulation/sec.: ○ = 76, x = 96, ● = 128 (2 tests), ▲ = 160, ■ = 192, Δ = 224.

responses to fall short of the expected linear relationship for small numbers of stimuli. We believe this is due to an intrinsic limitation of the method of measurement, since results such as those shown in figure 5 demonstrate that tension cannot be used as a measure of response frequencies which exceed some 50/sec. The relation-

ship described above seems to break down if the rate of stimulation of the nerve falls much below 40/sec., since in two experiments no block could be demonstrated with 1500 stimuli at this frequency.

#### DISCUSSION

The object of the experiments which we have described was to discover whether neuromuscular block occurred when the motor nerve to a mammalian muscle was stimulated repeatedly and to obtain some quantitative assessment of the part played in fatigue by failure of transmission. In our experiments we used frequencies of stimulation far below those known to cause Wedensky block, and they were generally within the frequency range of the discharge of motoneurons in voluntary and reflex contractions (Adrian & Bronk 1928, 1929). We have been able to demonstrate the occurrence of neuromuscular block with frequencies of nerve stimulation between 40 and 250/sec., and block has been shown to exist before there is any decline in tetanic tension. The technique which we have adopted has enabled us to assess neuromuscular block in terms of the frequency of response of the muscle fibres to the nerve impulses reaching them. No direct comparison between our results and those quoted in the introduction is possible. In earlier work, the measurement of neuromuscular block has usually depended upon the interpolation of single stimuli applied directly to the muscle, during the course of a tetanic contraction evoked by repeated excitation of the nerve (Davis & Davis 1932; Rosenblueth & Morison 1937). The interpretation of such experiments is made extremely difficult because the time relation of the direct stimulus to the arrival of impulses from the nerve is not known. Without a knowledge of these time relations it would be impossible to predict the tension of the twitch superimposed on the tetanic tension curve, even if the exact number of blocked fibres were known.

One of the most surprising features of our experimental results has been the finding that the response frequency of the partially blocked fibres of a fatigued nerve-muscle preparation is determined solely by the number of nerve impulses dispatched towards the muscle. This holds for all frequencies of stimulation at which block is demonstrable. For the purpose of this discussion it might be simpler to assume that the block occurs at the neuromuscular junction proper, but we would emphasize the fact that we have no evidence pointing to any specific location. The assumption has frequently been made in the past that neuromuscular block in fatigue is caused solely by reduction of the available stock of transmitter. In our opinion our results cannot be explained on such a simple hypothesis; if any fibre ceased to respond because the amount of transmitter liberated with each nerve impulse had fallen below the threshold for excitation there seems no reason that it should ever respond again. Our results show that complete block is certainly not the dominant phenomenon and probably never occurs.

It is clear that during tetanic stimulation, each successive nerve volley increases the refractory period of some peripheral part of the system, and because of this the proportion of blocked fibres steadily increases. The fact that the response frequency of muscle fibres after 1000 nerve volleys have impinged on the system, is

independent of their frequency of arrival, seems to imply that the time of recovery from the blocked state must be long in comparison with the interval between stimuli at the frequencies of tetanic stimulation we have used. We can only speculate on the site of these changes in refractory period; if the neuromuscular junction is concerned, the refractory period must be determined by the time taken for the product of the amount of transmitter liberated by each nerve impulse and the excitability of the motor-end plate, to reach threshold value. Our results would then imply that the return of this product to threshold value was comparatively slow and was not prevented by the arrival at the nerve terminals of impulses which were transmitted no further.

Another feature of our results which is of particular interest is the finding that muscle fibres which have been rested by neuromuscular block can, when they do contract, make a contribution to total tension greatly in excess of their contribution when unfatigued. Inspection of figure 8 shows that, after the preparation had received 1000 stimuli at 100/sec., the average response frequency of the muscle was 25/sec., implying that at any moment only one-quarter of the fibres of the muscle were responding. Figure 2 shows that at this moment, the tetanic tension had fallen only to 50 % of its original value, and it is obvious that one muscle fibre was now contributing with each response twice as much (tension  $\times$  time) as it did at the beginning of the tetanus, and four times as much per response as would a fibre responding throughout to every nerve volley. Bronk (1930) and Hill (1931) have shown that the efficiency of the single twitch of a frog's sartorius may be increased as much as five-fold by fatigue. This increase in efficiency is the result of an increase in the duration of the twitch and can clearly account for the improved performance in our experiments. We have evidence that recovery of tension from fatigue in our experiments was extremely rapid, and it is possible that another, different factor was responsible for the improved performance of fibres rested by neuromuscular block. This is the improvement of twitch tension which results from a tetanus, when an increase of tension  $\times$  time is brought about by an increase of peak tension, the duration of the twitch being the same or even less (Feng 1938; Brown & Euler 1938).

Whatever the precise nature of these changes, the increased tension-time which a fibre develops after it has been rested by block, is sufficient to compensate almost exactly for the loss of tension due to its failure to respond to every nerve impulse. Our experiments have shown that, although neuromuscular block regularly occurs at frequencies of nerve stimulation which are within the physiological range, block cannot be regarded as being in any way responsible for the fall of tension which occurs as the system fatigues.

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## The heat of activation and the heat of shortening in a muscle twitch

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The heat produced in a single muscle twitch is made up of two parts, (1) the heat of activation, and (2) the heat of shortening. In the leg muscles of toad or frog at 0°C the heat per cm. of shortening is about 350 g.cm. (expressed in mechanical units) per sq. cm. of muscle cross-section. The heat of activation is usually rather less than the maximum heat of shortening and depends little, if at all, on the length at which the stimulus was applied or on changes of length thereafter: it is equal to the heat which would be produced if shortening were altogether obviated, a condition approximately realized in a muscle brought to a very short length by previous stimulation under a very small load.

The heat of shortening occurs at the same time as the shortening. The heat of activation has its maximum rate at the start, very soon after the stimulus, and falls off in rate from then onwards.

The heat of maintenance in a tetanic contraction is the summated effect of the heats of activation resulting from successive elements of the stimulus.

The effect on the heat production of a sudden arrest of an isotonic contraction is described. Under such conditions the contractile elements of a muscle continue to shorten but at a decreasing rate as the tension rises.

The complications due to inequalities of length and contractility in the different fibres of a muscle are discussed.

Previous experiments on the heat of shortening of muscle (Hill 1938) were made on tetanic contractions. The equipment was not sensitive or rapid enough to give reliable results in single twitches. With the galvanometers recently constructed (Hill 1948*a, b, c*; Downing 1948) ample sensitivity is now available, at a speed of deflexion giving only about 2 msec. lag in recording a varying current. The thermopiles are unchanged, except in details of their use, but the very rapid galvanometer has led to a much better understanding of their properties and they now provide records which allow most of the characteristics of the heat production to be exhibited directly without any analysis. For the finer quantitative study of some of these an analysis is still required, to obviate the effects of heat flow and heat loss: but this can now be made much simpler and less laborious than it was.

It was possible that some of the characteristics of the heat of shortening might be due to, or affected by, the fact that the shortening investigated occurred while

the tetanic stimulus was going on. In any case, the single twitch is the elementary unit of all muscular response, and it was very desirable to know how the heat of shortening behaved during a twitch, whether the heat had the same quantitative relation to the shortening as in a tetanus, and whether mechanical work appeared as an 'extra' when a load was provided during the shortening. The chief difficulty, apart from the requirements of speed and sensitivity, in investigating the single twitch is the fact that relaxation sets in before the muscle can shorten its full amount, even under a very light load, and before it can develop its full tension isometrically. The experiments, therefore, are essentially dynamic, the properties of the muscle have to be investigated during the short interval between a shock and the moment when the contraction begins to be dissipated in relaxation. It is satisfactory, therefore, that some strikingly simple relations have been found, throwing far more light on the properties of muscle than experiments on tetanic contractions alone could have done.

Let us consider first the evidence that the heat of shortening does appear as a recognizable entity in a twitch. A muscle of uniform cross-section, lying on a thermopile, is connected to an isotonic lever by a chain. The thread joining it to the chain is short, the chain is vertical and practically inextensible. The carriage of the lever is provided with an adjustable stop, so that shortening can be arrested suddenly at any stage. The muscle is stimulated by a single short condenser discharge, the cathode being near the middle, anodes being near the ends. There is no afterload, so that the muscle is under the same tension throughout an unarrested contraction: consequently the elastic structures in series with the contractile ones are of constant length, while the shortening of the contractile elements is measured, without distortion, at the lever. When the lever is arrested by the stop the contraction suddenly becomes isometric, the tension begins to rise and any elastic structures in series with the contractile ones (thread, tendons, non-contractile regions of the fibres, etc.) begin to be stretched. The rate of shortening  $v$  of the contractile structures is not affected immediately by the arrest:  $v$  depends on the tension  $P$ , according to the characteristic equation (Hill 1938) or some modification of it appropriate to a single twitch, and  $P$  rises not suddenly but gradually when the lever is arrested: the instantaneous effect of the arrest is to alter  $dv/dt$ , not  $v$ . As  $P$  rises, the rate of shortening of the contractile elements, and of lengthening of the elastic ones, decreases: but the final state is never reached in a twitch, before relaxation sets in. The only way to stop the shortening abruptly would be to apply suddenly an extra opposing force of the appropriate strength: but since that strength is unknown the method could not be used quantitatively.

If a muscle consisted only of contractile material, without any elastic elements in series with it, and were of uniform strength throughout its length, it would be easy to demonstrate the heat of shortening by comparing the heat when shortening was permitted with that at constant length. In fact, however, under the most rigidly isometric conditions the contractile elements can shorten a considerable amount (5 to 10 % of their length) by stretching the elastic elements; and moreover there is good evidence that muscle fibres are not of uniform strength throughout their length, nor are they excited all over at once, so that one region may extend



another when external shortening is prevented. The heat of shortening, therefore, must be measured under more sophisticated conditions, though its existence can be shown readily enough.

In figure 1 are two superimposed records, exactly as observed, of the heat produced in two consecutive twitches of a toad's muscle at 0°C. In one of them (hollow circles) shortening was unlimited, being in fact 8.5 mm. (40 % of the initial length). In the other (solid circles) it was limited by the stop to 2.08 mm. (10 %). The actual shortening is shown, ending abruptly at 0.27 sec. Up to that moment the two records are identical, from then on they diverge, slowly at first but then more rapidly as internal shortening in the arrested muscle becomes progressively slower under the rising tension.

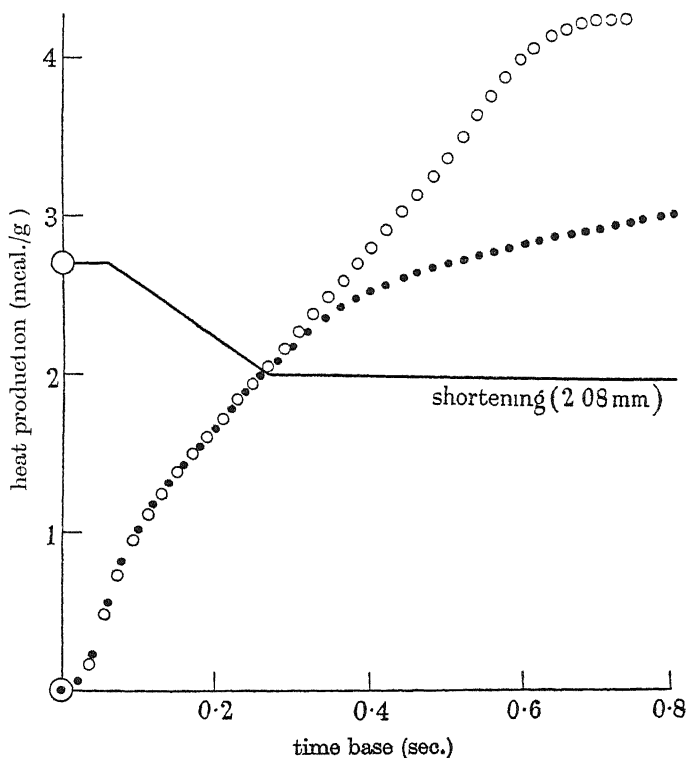


FIGURE 1. To show heat of shortening in single twitch. Two semi-membranosi of toad, 58 mg., isotonic load 4.32 g., 21 mm. long, 0°C, single shocks. Heat production vertically, scale as shown: time-base horizontally: time spots every 0.02 sec. Hollow circles, for isotonic shortening, unlimited. Solid circles, for shortening arrested after 2.08 mm. Shock and start of sweep at  $\odot$ . Solid line, shortening (arrested) recorded simultaneously (downwards) on the cathode-ray tube. Note that the superimposed records coincide up to 0.27 sec. when arrest occurred, then diverge as the arrested muscle gradually cease to shorten internally under rising tension.

In figure 2 six consecutive records at 5 min. intervals have been fitted together, one with unlimited shortening, one with arrest at each of the moments indicated. The most rapid divergence occurs with the latest arrest *E*—as was expected, since after 5.3 mm. (25 %) shortening the isometric force available to stretch the elastic

elements is much less. In fact, as the figure shows, the rate of divergence gets steadily greater for later arrest, corresponding to a smaller isometric force at a shorter length.

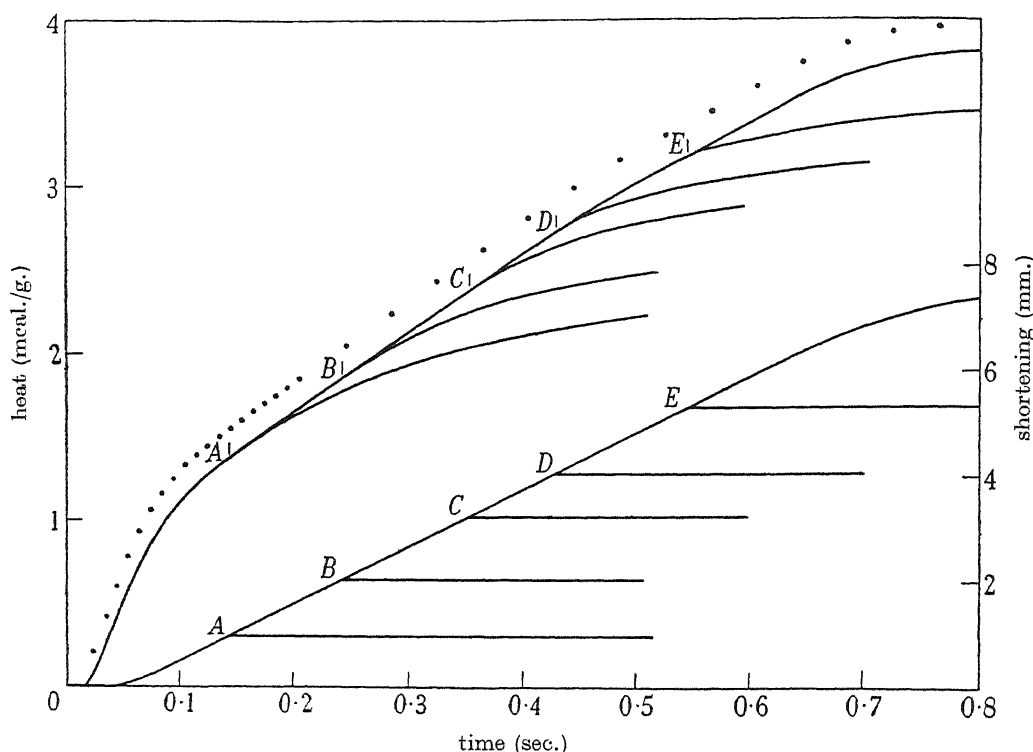


FIGURE 2. To show heat of shortening in single twitch. Two semi-membranosi of toad, 58 mg., isotonic load 3.46 g., 21 mm. long, 0°C, single shocks. Six records fitted together, upper curves heat production, lower curves shortening. Shortening (max. 8.3 mm.) was complete in about 1.1 sec. One contraction was unlimited, in the others a stop was introduced to arrest shortening at about 1, 2, 3, 4 and 5 mm., at A, B, C, D and E respectively. Each record coincides with the unlimited one until arrest occurs, then diverges owing to the gradual slowing up of internal shortening. For discussion, see text. Records as observed. The inertia of the galvanometer caused about 2 msec. lag in the recording, the thermopile introduced a thermal capacity equal to about  $22\mu$  of muscle. Allowance for these gave (for unlimited shortening) the result shown by the dots.

No correction has been applied to these curves to allow for lag due to heat-flow etc., they are exactly as recorded. Were a correction applied, the divergence would be slightly more rapid. To illustrate the effect of such allowance the analysis of the heat in the 'unlimited' contraction is shown by the dots in the figure.

Although figures 1 and 2 indicate beyond gainsay that extra heat is associated with shortening they provide no quantitative method of relating the two. The main reason for this is that the amount of internal shortening after arrest is unknown: a secondary reason is that, as the tension rises after arrest, one region of any muscle fibre is liable to pull out another weaker region, doing work on it part of which is degraded at once into heat. Thus, an unknown part of the heat observed

after arrest is not associated with net internal shortening at all, and even if we knew the latter we could not relate the difference of heat simply to the difference of shortening. We must avoid two complications alike (i) that due to the unknown redistribution of length between contractile and elastic structures as the tension varies, and (ii) that due to probable non-uniformity of strength along the length of individual muscle fibres.

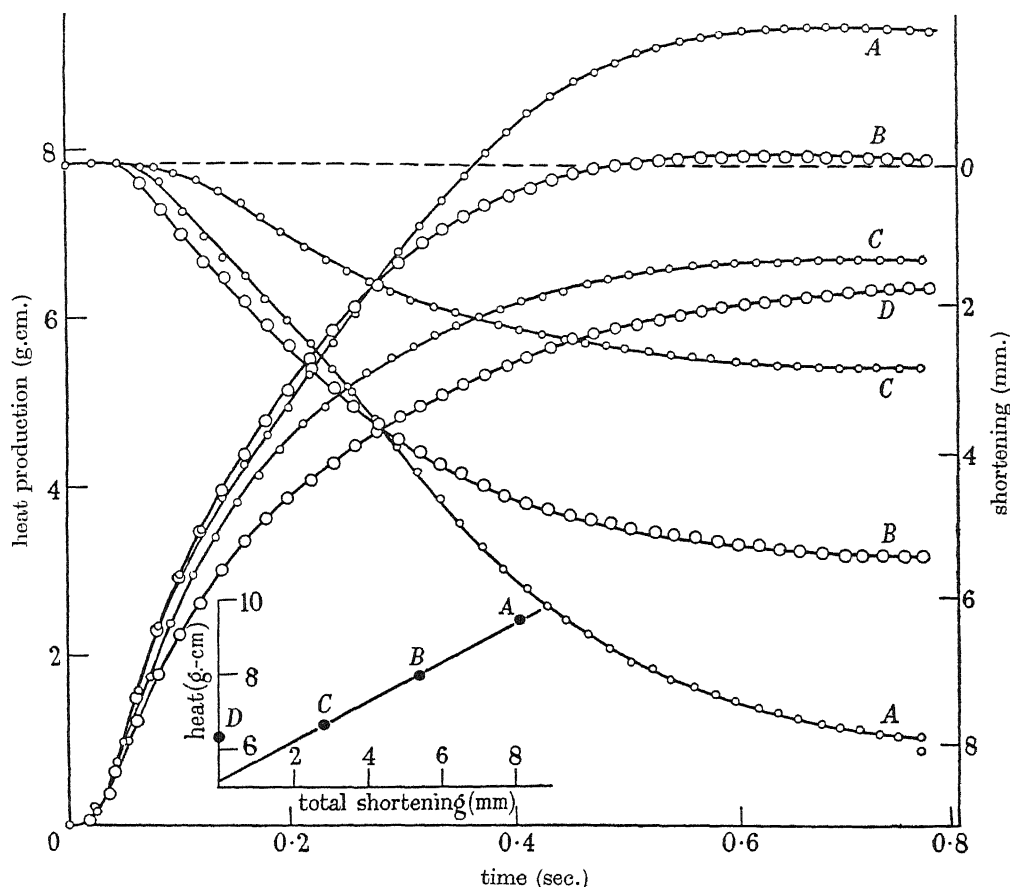


FIGURE 3. To show the effect of shortening on the heat production in a twitch. Superimposed records of heat and shortening, as observed, in four consecutive twitches. Time spots every 0.02 sec. Two semi-membranosi of toad, 58 mg.,  $0^{\circ}\text{C}$ , isotonic load 0.17 g. *D*, starting at length 9.5 mm. after three preliminary shocks: *C* pulled out to 12.5 mm.: *B* pulled out to 15.5 mm.: *A* pulled out to 18.5 mm. No shortening was visible in *D*. Small figure inset: total heat against total shortening, see text.

Figure 3 shows four consecutive records of the heat production (upwards) and the shortening (downwards) in isotonic twitches at  $0^{\circ}\text{C}$  of a pair of semi-membranosus muscles of a toad. The curves, made simultaneously of heat and shortening on a cathode-ray tube, have been superimposed exactly as recorded. The muscles were under an extremely small load, 0.17 g., and everything had been adjusted so as to avoid friction or 'sticking' as far as possible. The muscles, which were 21 mm. long under a load of 3.5 g. and consistently returned to that length after contrac-

tion, shortened passively to 18.5 mm. when the load was altered to 0.17 g. When stimulated by a single shock under this load the muscles shortened 7 or 8 mm. and practically no visible relaxation occurred afterwards. Two more shocks further reduced the resting length to 9.5 mm. At this length a single shock was applied and curve *D* of the heat production recorded: no visible shortening occurred. The muscles were then gently pulled out to 12.5 mm. and remained at that length under 0.17 g. A shock now applied gave records *C* of heat and shortening, the muscles returning to nearly 9.5 mm. They were then pulled out gently to 15.5 mm. and remained at that length under 0.17 g.: a shock gave records *B*. Finally, they were pulled out gently to 19.5 mm. and returned passively to 18.5 mm. under 0.17 g.: a shock gave records *A*.

The total heat and the total shortening recorded in the four twitches were as follows:

twitch	<i>D</i>	<i>C</i>	<i>B</i>	<i>A</i>
initial length (mm.)	9.5	12.5	15.5	18.5
shortening (mm.)	0	2.8	5.4	8.1
heat (g.cm.)	6.4	6.7	8.0	9.5

Heat has been plotted against shortening in the small figure inset. Neglecting *D* for the moment, a linear relation is found between the two, having the following characteristics:

	g.cm.
extra heat per cm. shortening	5.3
heat for zero shortening	5.2

In the 1938 paper the heat of shortening was expressed per cm. of shortening per sq. cm. of muscle cross-section. The values found for the tetanic contraction of a frog's sartorius averaged about 400 g.cm. The cross-section was reckoned for a moderate load on the muscle. In the present experiment the muscles weighed 58 mg. and were 21 mm. long under a load of 3.5 g.: the calculated cross-section is 2.6 sq. mm. Reckoned, therefore, per sq. cm. of cross-section, the extra heat per cm. of shortening is 204 g.cm. This is smaller than the 400 g.cm. found for the tetanic contraction of the frog's sartorius.

The heat in contraction *D* is greater than would correspond to the absence of visible shortening. There is no certainty, however, that *all* the fibres of the muscle remained at 9.5 mm. after contraction; what we know is that some of them did and were able to hold the very small load: others may have relaxed and become loose. If so, these latter fibres would shorten and straighten when a shock was applied and the average shortening would not be zero but perhaps 2 or 3 mm. The same consideration might apply, though to a much smaller degree, in the twitch starting at 12.5 mm. In the small inset figure, therefore, point *D* ought to be shifted considerably to the right, *C* a smaller amount, *B* a still smaller amount and *A* not at all. The net result of this might be to put *D* on the line, somewhat to increase the slope of the line and therefore the heat of shortening per cm., and somewhat to decrease the heat for zero shortening.

The method just described, although providing strong qualitative evidence of the heat of shortening, cannot yield accurate quantitative values of it since there

is no certainty that all the fibres are involved in the whole of the observed shortening. If, under a very small load, the observed shortening from the greater (pulled-out) lengths were greater than the average shortening of the fibres, while from the smaller lengths (for the reason just given) it were less, the calculated heat of

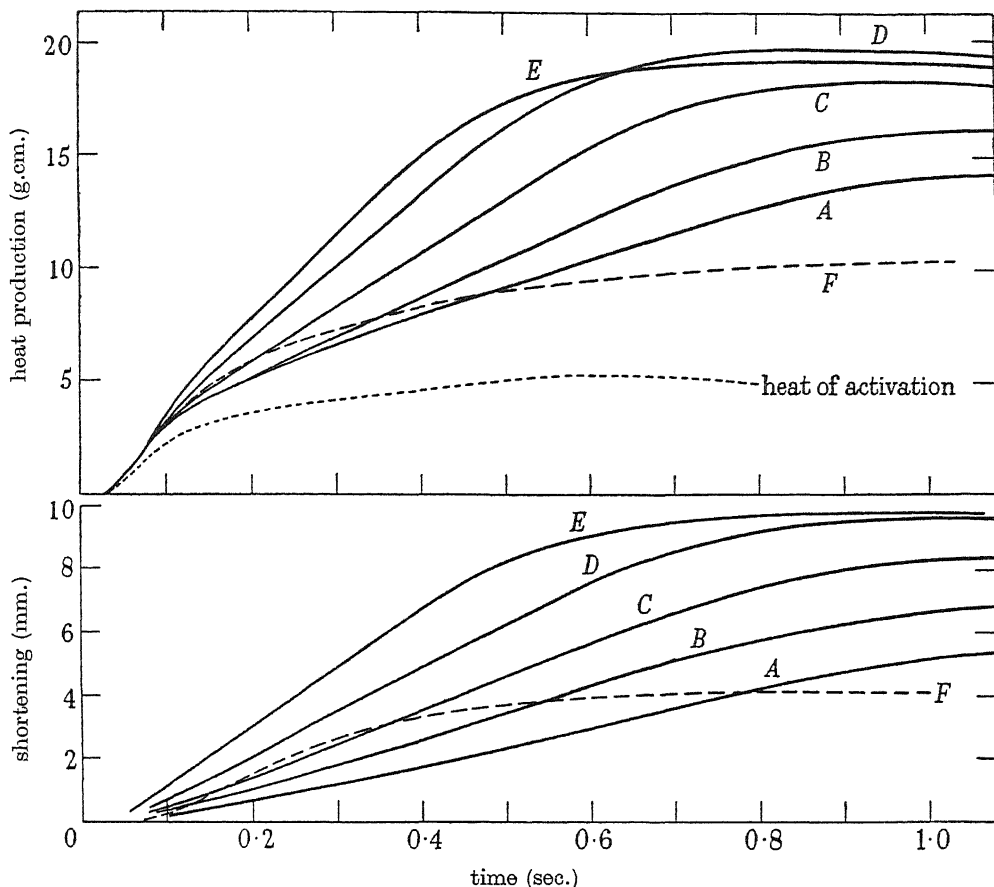


FIGURE 4. To show heat of shortening and heat of activation in a twitch. Lower curves shortening, upper curves heat, in six successive twitches of a pair of toad's semi-membranosus muscles ( $103\frac{1}{2}$  mg.) at  $0^{\circ}\text{C}$  under various isotonic loads. *A*, 17.3 g., 26 mm. *B*, 13.0 g., 25.7 mm. *C*, 8.65 g., 25.0 mm. *D*, 4.32 g., 23.5 mm. *E*, 0.86 g., 20.3 mm. *F*, 0.86 g., 13.5 mm. No afterloading. Records as observed, no averaging, no allowance for heat loss, no analysis. Heat in mechanical units (g.cm.): to express in mcal. per gram divide by 4.38. Relaxation (not shown) began at 1.3 to 1.4 sec. after which, particularly with the greater loads, there was a large heat deflexion derived from the mechanical work dissipated. The heat of activation was calculated as described in the text.

*Note.* The mechanical efficiency can be calculated from the load, the shortening and the heat as follows: *A*, 40%; *B*, 35%; *C*, 29%; *D*, 17%; *E*, 4%. This assumes that the elastic energy under any load is the same at the height of the contraction as at the start.

shortening per cm. would be too low. It is, in fact, considerably too low. A better method, therefore, is required. In figure 4 are the records of six consecutive isotonic twitches, heat and shortening, of a pair of toad's semi-membranosus muscles at  $0^{\circ}\text{C}$ . Maximal shocks were applied every 6 min. and the muscles were in a steady

state. Five different loads were used, from 17.3 to 0.86 g.; there was no afterload. Under each load in turn the muscles were given 2 or 3 min. to settle before the next stimulus; the length at which each stimulus was applied is noted in the legend. After *E*, stimulated under a very small load, the length of the muscles was much less than before, and contraction *F*, under the same load as *E*, occurred from a much shorter initial length.

A comparison of the curves of heat and shortening shows at once how closely they are related: the greater the shortening the greater the heat, the more rapid the shortening the quicker the heat production. The apparent slight exception in the comparison of *D* and *E* is readily explained; the muscle in *E* started 3.2 mm. shorter than in *D* and shortened so much under the very small load (down to 10.6 mm.) that many of the fibres probably reached their limit of shortening well before the end, so that the *average* shortening was substantially less than that observed externally. The heat, being determined by the average shortening of the fibres, not by the maximum shortening of some of them, is rather less than would correspond to the latter.

Records *F* for heat and shortening, under very small load and from very short initial length, show the behaviour described in connexion with figure 3 above. The intermediate early rate of shortening, and the smaller total shortening, correspond to similar characteristics of the heat. Less or slower shortening results in less or slower heat production, whether due to starting at a very short length or to shortening under a heavy load.

A simple relation obviously exists between heat and shortening. They are not, however, directly proportional to one another; the observed heat includes a quantity which we may call the 'heat of activation', which is what we should observe on stimulation if shortening could be completely obviated.\* Isometric conditions, as we have seen above, do not prevent the contractile elements of a fibre from stretching the passive elastic elements in series with them, or other weaker contractile regions. Stimulating a muscle under a very small load may cause it to shorten so much that no visible external shortening results from a later stimulus (compare figure 3, record *D*) but internal shortening of some of the fibres probably still occurs making the heat observed greater than the 'heat of activation'. It is

\* There may be some danger of confusing the term 'heat of activation' with the 'energy of activation' *E* calculated from the effect of temperature on the velocity *k* of a chemical reaction according to the Arrhenius equation  $d \ln k/dT = E/RT^2$  (see, for example, Höber 1945, pp. 32, 387 etc.). It is necessary, therefore, to make clear that no hypothesis of any connexion between the two is implied in the use of the term: it is intended only to denote the heat which a muscle would give out in a single twitch, as a consequence of passing through a single cycle of activity, if shortening, internal as well as external, of its contractile component could be completely obviated. In principle this condition might be satisfied by stretching and releasing a muscle during a twitch at such a rate that the length of its contractile component remained constant throughout. No heat of shortening could then occur. In practice, however, the procedure would be difficult to apply and there could be no certainty that the length had really remained constant. By measuring the heat of shortening independently and subtracting it from the whole heat we are left with a remainder which seems to be connected with nothing except the fact that the muscle has passed through a cycle of activity. The term 'heat of activation' has been adopted with some hesitation because of the danger of confusion, but no other seems to describe the phenomenon nearly so aptly.

possible, at the other extreme, to load a muscle so heavily that it practically cannot shorten or develop a force at all. According to Hill (1925) 'at high initial extensions, of the order of 50 %, the heat is about 60 to 70 % of its maximum value, while the tension has practically disappeared'. According to Ramsey & Street (1940), the isometric tension of a single fibre falls practically to zero at about 100 % extension. Stretching a whole muscle so far, however, is apt to injure it so much that reproducible results are not obtained, while the technical difficulty of measuring heat in a single fibre is too great to be contemplated.

It is impossible, therefore, to measure the 'heat of activation' directly. It must be obtained indirectly as a difference. Its existence, however, is shown very clearly by the way in which the heat curve, e.g. in figure 2, rises much more rapidly at first than later on, and more rapidly than the curve of shortening. To anticipate later results, the 'heat of activation' begins at its maximum rate soon after the stimulus, before shortening is detectable by ordinary methods, then diminishes in rate continually as contraction proceeds. The observed heat is the sum of the 'heat of activation' and the heat of shortening.

If we assume that the amount and the time course of the heat of activation are independent of the length at which stimulation occurred, and of subsequent changes of length, we can clearly eliminate it by subtracting the ordinates of two heat curves (e.g. *A* and *D*, figure 4) and comparing the differences  $\Delta H$  with the differences of shortening  $\Delta S$  at corresponding times. The ratio  $\Delta H/\Delta S$  should then be constant. The following results, calculated from the curves of figure 4 over the range 0.24 to 0.76 sec., show that it is approximately constant and has a mean value of about 17.2 g.cm. of heat per cm. of shortening. The value of  $\Delta H/\Delta S$  is obviously not exactly constant, there are consistent slight variations: those might be due either to the heat of activation not being completely independent of length, or to the heat of shortening per cm. not being quite the same at different lengths, or to some other cause. The muscles weighed 103.5 mg. and were 23.5 mm. long under a load of 4.3 g.: their cross-section therefore, can be taken as 0.042 sq.cm. Reckoned per sq. cm. of muscle cross-section, the heat of shortening per cm. then comes to  $17.2/0.042 = 410$  g.cm. This is very close to the average value, 400 g.cm., found in the tetanic contraction of a frog's sartorius (Hill 1938).

*Values of  $\Delta H/\Delta S$  (g.cm. per cm.)*

time (sec.)	0.24	0.28	0.32	0.36	0.40	0.44	0.48
<i>D-A</i>	15	16	16	16	16	18	18
<i>D-B</i>	18	19	20	20	20	22	21
<i>C-A</i>	13	13	15	16	16	17	17
time (sec.)	0.52	0.56	0.60	0.64	0.68	0.72	0.76
<i>D-A</i>	18	17	17	16	16	15	15
<i>D-B</i>	20	19	19	18	17	16	15
<i>C-A</i>	17	18	18	18	18	18	17

We are now ready to calculate the heat of activation. In any contraction, subtract from the observed heat at any moment the heat of shortening reckoned as the observed shortening (in cm.) up to that moment multiplied by 17.2: do this all along the curve. The result plotted against time gives the amount and course of

the heat of activation. The dotted curve at the bottom of figure 4 was calculated as a mean from records *A*, *B*, *C* and *D*. It is not, probably, very exact because the precise accuracy of the assumptions made in calculating it is not certain. It gives, however, a clear indication of the general character and extent of the heat of activation in a twitch.

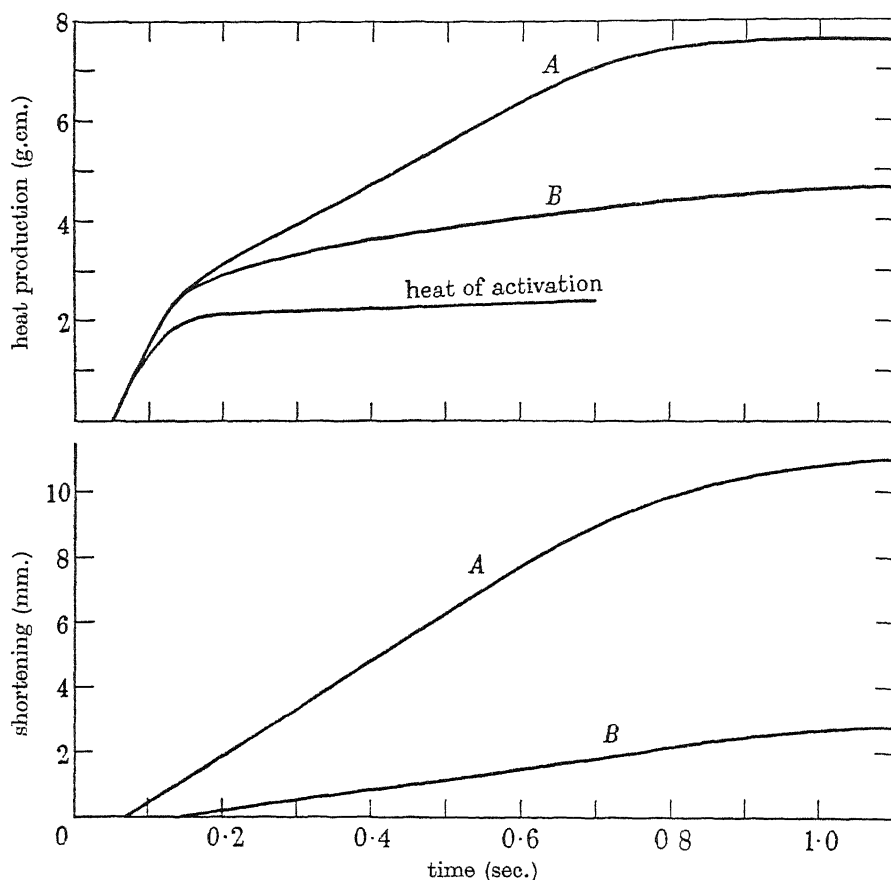


FIGURE 5. To show heat of shortening and heat of activation in isotonic twitches. Two sartorii of toad, 61.7 mg., 2.6 g. initial load, 32 mm. long, 0°C. *A*, no afterload; *B*, 8.6 g. afterload. Mean records of heat and shortening in the series *B*, *A*, *B*, *A*, *B* at 5 min. intervals. Heat records corrected for heat loss, not otherwise analyzed. Heat of activation calculated as in the text.

It is possible to avoid any assumption that the heat of activation is independent of initial length by working with afterloaded contractions starting always at the same length, and the most reliable results have been obtained by so doing. We cannot, however, then assume that the observed difference of shortening  $\Delta S$  is the actual difference, since the contractile portion of the muscle has shortened some unknown amount in extending the elastic portion before the afterload is lifted. Instead of expecting to find  $\Delta H/\Delta S$  constant we should now expect  $\Delta H$  to be a linear function of  $\Delta S$ , the slope of the line giving the heat of shortening per cm. In figure 5 are mean records (heat and shortening) of a pair of toad's sartorii in



isotonic twitches with the same initial load 2.6 g., *A* without afterload, *B* with 8.6 g. afterload.

In figure 6  $\Delta H$  is plotted against  $\Delta S$  at intervals of 0.05 sec., from the moment when the afterload in *B* was just beginning to be lifted to the end of the contraction. Over nearly the whole range of shortening the relation is linear, corresponding to a heat of shortening per cm. of 5.25 g.cm. The cross-section of the muscles calculated from weight and length under a small load was 1.84 sq. mm. so that the heat of shortening per cm. per sq. cm. of muscle cross-section was 290 g.cm. The isometric tension in this experiment was about 22 g. so that  $a/P_0$  was about 0.24.

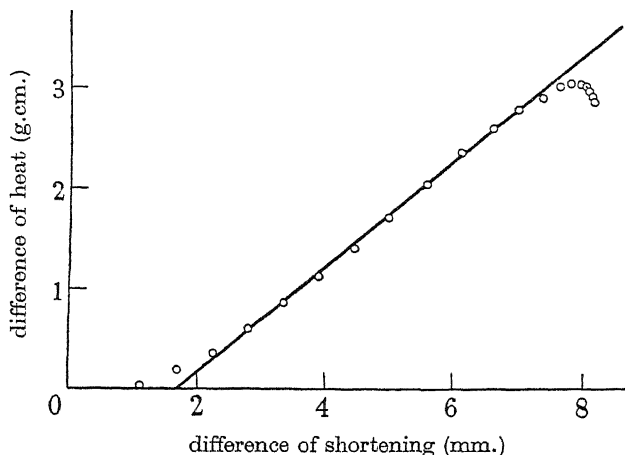


FIGURE 6. Calculation of heat of shortening from curves *A* and *B* of figure 5. Differences of heat ( $\Delta H$ ) plotted against differences of shortening ( $\Delta S$ ) at corresponding times, from 0.15 sec. to 1.1 sec. Slope of linear portion of line, 5.25 g.cm. per cm. shortening.

The heat of activation was calculated from the heat in record *A*, by subtracting the heat of shortening obtained by multiplying the observed shortening by 5.25. It is shown in figure 5.

The relation between  $\Delta H$  and  $\Delta S$  deviates from linearity after about 0.7 sec., by when the shortening with the smaller load is about 80 % complete. One reason for this may be as follows. Some of the muscle fibres may have reached the limit of their shortening by then, so that progressively thereafter the average shortening of all the fibres was less than the observed shortening, and the scale of  $\Delta S$  at the end ought to be compressed. This would preserve the linear relation rather longer, but it cannot explain the drop of  $\Delta H$  at the end. That may imply that the heat of shortening per cm. is slightly greater when the muscle is longer than when it is shorter—at 0.8 sec. we are comparing the muscle about 22 mm. long with itself when about 30 mm. long. It would be no wonder if the heat of shortening were somewhat different at different lengths, it is surprising rather that the linear relation between  $\Delta H$  and  $\Delta S$  is maintained over so great a range.

The results given hitherto have been obtained from records of heat as observed, without analysis, i.e. without allowance for delay due to the inertia of the galvanometer or the heat capacity of the thermopile. The instruments are indeed very

rapid, but the results are slightly improved by making such allowance and the heat curves in figure 7 were obtained by analysing the records up to 0.9 sec. Afterloads were over a wide range, from zero up to almost the largest the muscles could lift. Figure 8 shows the relation between  $\Delta H$  and  $\Delta S$  obtained by comparing 6 pairs of curves. The slopes were practically the same for all of them, and had a mean value of 15.5 g.cm. per cm. shortening, or reckoned per sq. cm. of muscle cross-section, about 360 g.cm. The value of  $a/P_0$  was about 0.32. The heat of activation was similar to that given in previous figures, having a maximum value of about 7 g.cm., i.e. about one-third of the total heat with the greatest shortening.

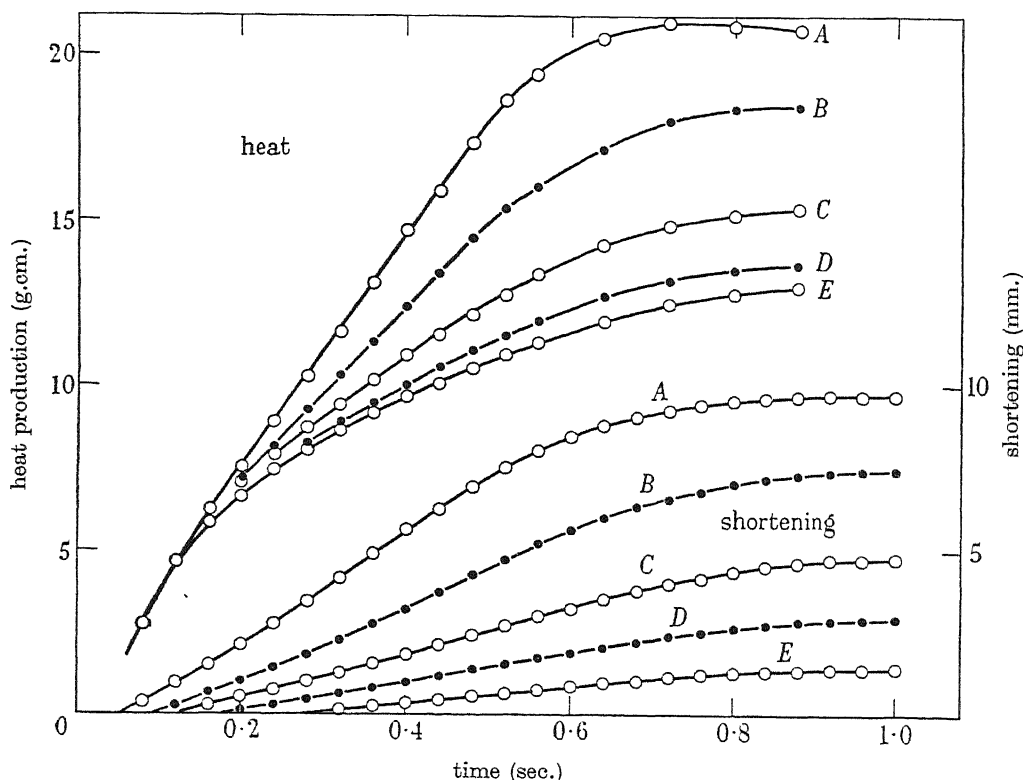


FIGURE 7. To show heat of shortening in isotonic twitches. Two semi-membranosi of toad, 116 mg., 2.6 g. initial load, 25.5 mm. long, 0°C. Afterloads as follows: A, none; B, 6.05 g.; C, 15.7 g.; D, 23.4 g.; E, 32.0 g. Means of heat and shortening in the series A, B, C, D, E; E, D, C, B, A. Allowance made in heat records for heat loss and for lag due to galvanometer and thermopile: results of heat analysis shown by circles.

The results described hitherto were obtained on the muscles of toads (English *Bufo bufo*) which are singularly good for such experiments, being very slow, contracting strongly and consistently and shortening considerably more than frogs' muscles. Several experiments have been made on the muscles of American toads (*B. americanus*), sent to me by the kindness of Dr Paul Weiss, but these are as fast as those of English frogs, and shorten not more but less. It is curious that animals so alike in size and anatomy as English and American toads should differ so largely

in speed and shortening. Possibly the difference is connected with the fact that American toads jump much better than English toads, the latter being more adjusted to crawling and climbing. Anyhow, far the best material found hitherto for myothermic experiments is the leg muscle of the English toad. Since, however, most such experiments in the past have been made on frog muscle, one experiment of the present series on the sartorii of an English frog (*Rana temporaria*) is included here to show that similar results are obtained. A comparison indicates that the time scale of the toad's muscle is about twice that of the frog's, which makes the instruments relatively twice as rapid and the results more accurate and reliable.

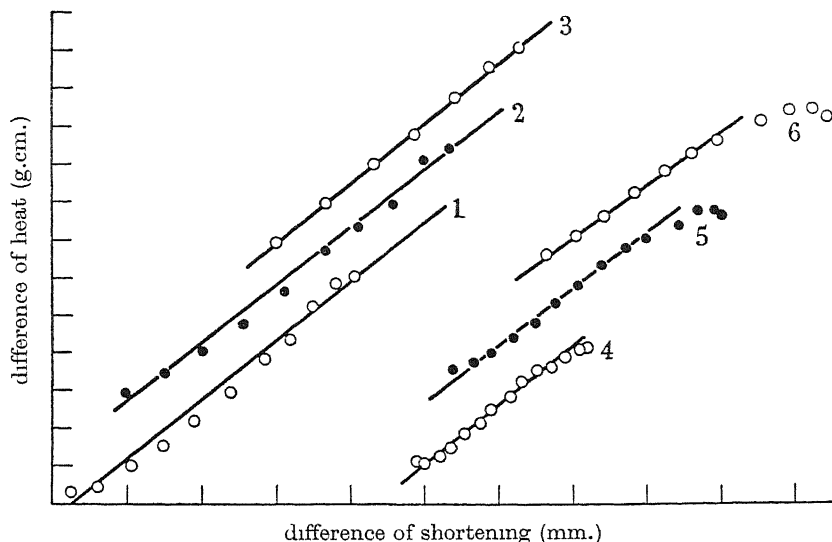


FIGURE 8. Calculation of heat of shortening from curves *A* to *E* of figure 7. Differences of heat ( $\Delta H$ ) plotted against differences of shortening ( $\Delta S$ ) at corresponding times: (1) for *A* and *C*, (2) for *A* and *D*, (3) for *A* and *E*, (4) for *B* and *C*, (5) for *B* and *D*, (6) for *B* and *E*. Mean slope of line, 15.5 g.cm. per cm. shortening.

The heat curves in figure 9 on the sartorii of a frog were obtained from the mean records by analysis. The analysis is much more important than with the slower muscles of a toad. Exactly similar results are obtained on a different scale of time. In figure 10  $\Delta H$  is plotted against  $\Delta S$  for three pairs of records of figure 9. The slopes are identical, corresponding to 8.9 g.cm. per cm. of shortening, or 2.40 reckoned per sq. cm. of muscle cross-section.  $a/P_0$  was about 0.22. The heat of activation was calculated as before and is given as curve *E* in figure 9. For comparison is included curve *F*, which is the actual heat given out in a contraction under 0.4 g. load from an initial length 12 mm. less than in *A* to *D*. The muscle had been reduced to this length by three previous shocks under 0.4 g. and showed only 0.3 mm. of visible shortening when stimulated. The fibres had shortened so far that little further shortening was possible, and the heat of shortening had been reduced practically to nothing.

In all, eight reliable experiments of this kind were made, the mean value of the heat of shortening per cm., reckoned per sq. cm. of muscle cross-section, being

350 g.cm. The isometric tension was not always measured, the mean value of  $a/P_0$  when it was being about  $\frac{1}{4}$ . In a single twitch relaxation occurs too soon to allow the full isometric tension to be developed (see the curves of Hill 1938, p. 187), so that the real value of  $a/P_0$  was probably rather less than  $\frac{1}{4}$ . These quantities are in reasonably good agreement with the mean values found in 1938 for the tetanic contraction of a frog's muscle, viz. 400 and  $\frac{1}{4}$  respectively.

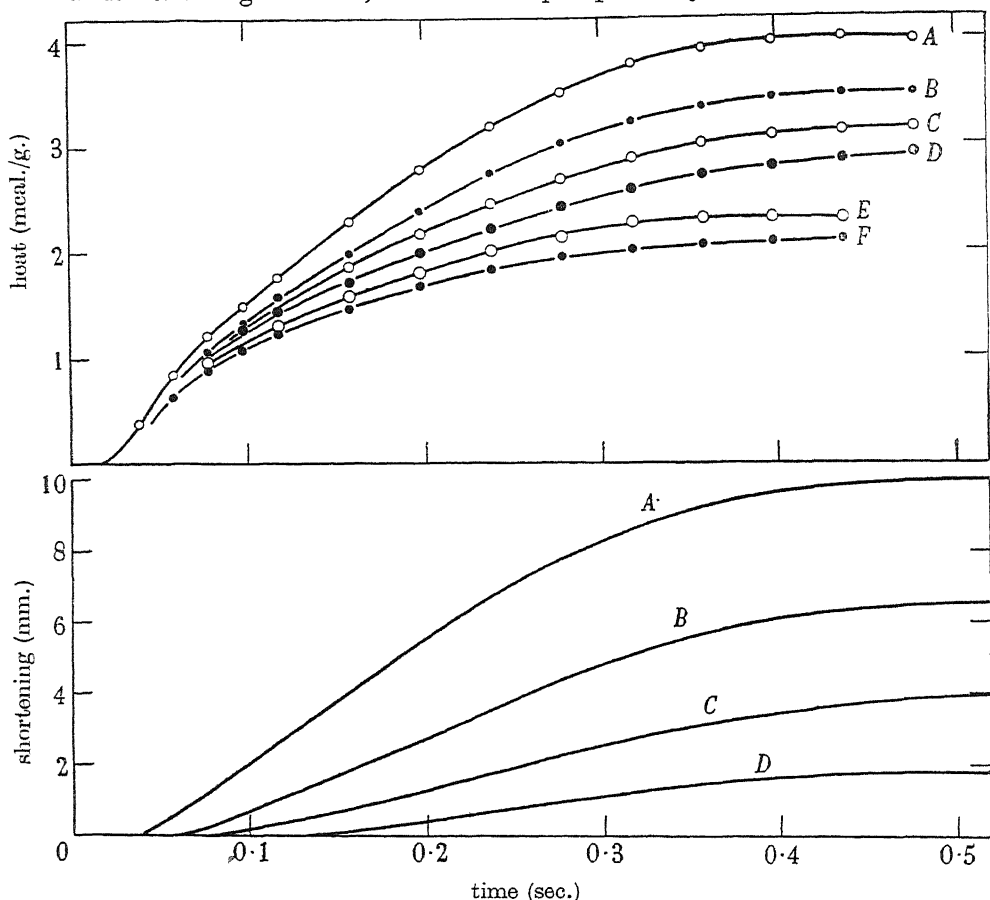


FIGURE 9. To show heat of shortening and heat of activation in isotonic twitches. Two sartorii of frog, 120.5 mg., 1.73 g. initial load, 31 mm. long, 0°C. Afterloads as follows: A, none; B, 6.92 g.; C, 15.6 g.; D, 24.3 g. Means of heat and shortening in the series A, B, C, D; D, C, B, A. Allowance made in heat records for heat loss and for lag due to galvanometer and thermopile: results shown by circles. Curve E is the calculated heat of activation, curve F is the heat observed when the muscles were stimulated under 0.4 g. load after previous shocks had reduced their length to 19 mm.

It will have been realized that the results given above must have demanded considerable accuracy in the records of heat and shortening, particularly since much of the argument has required differences to be taken between quantities not widely unequal. To show that the required accuracy is, in fact, obtainable, two actual records of heat and shortening are given in figure 11. The spots show the instantaneous value of the deflexion 50 times per second, heat upwards, shortening

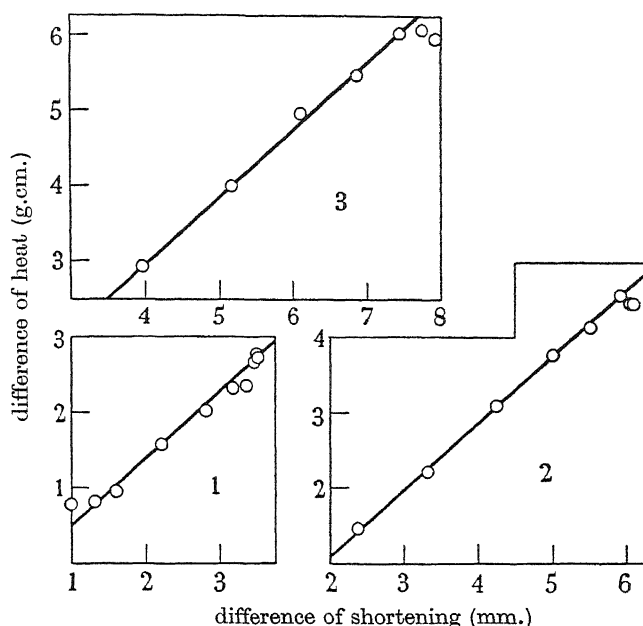


FIGURE 10. Calculation of heat of shortening for curves *A* to *D*, figure 9. Differences of heat ( $\Delta H$ ) plotted against differences of shortening ( $\Delta S$ ) at corresponding times: (1) for *A* and *B*, (2) for *A* and *C*, (3) for *A* and *D*. Slope of line in each, 8.9 g.cm. per cm. shortening.

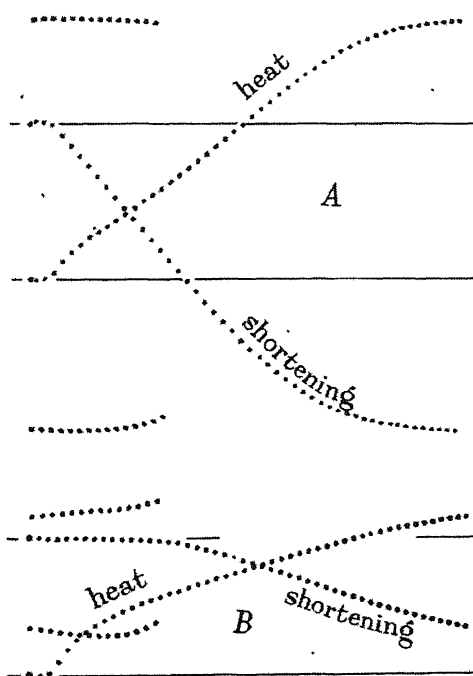


FIGURE 11. Actual records of heat and shortening, toad's muscles, 0°C. Isotonic contractions, initial load 6 g.; afterload, *A* none, *B* 37.2 g. Heat up, shortening down. Time spots every 0.02 sec. The spots on the left show the moment of the stimulus and the start of the sweep. Base lines drawn in, records otherwise unaltered.

downwards; the stimulus was applied and the sweep started at the spot on the left. Contractions were isotonic with 6 g. initial load. In *A*, there was no afterload, in *B* 37.2 g. In *A*, the first time spot was at 10 msec. and the record went to 1250 msec.; in *B*, the first time spot was at 20 msec. and the record went to 1240 msec. The records have not been altered in any way, apart from base lines being drawn in. They were read to 0.1 mm.

### DISCUSSION

From previous work (e.g. Hill 1939) on the heat produced in a tetanic contraction, it was necessary to assume that heat was produced, quite independently of shortening or work, in the process by which a contraction is maintained. It may be concluded now that the 'maintenance heat' in a tetanus is nothing more than the summated effect of the 'activation heat' produced in response to each of the series of shocks composing the tetanus. It will be shown in a later paper that the activation heat begins very soon after a stimulus, its maximum rate being at the start like the discharge of a condenser through a resistance. Of the nature of the physical or chemical processes underlying it we have at present no evidence, but it represents presumably a 'triggered' reaction setting the muscle in a state in which it can shorten and do work. If it shortens, extra heat is given out in proportion to the amount of shortening; if it does work, extra energy is mobilized to provide the work, but, for a given amount of shortening, the heat is unaffected. Whether the heat of activation, the heat of shortening and the work are derived from different physical or chemical sources we cannot yet say: but they can be so sharply distinguished from one another that there is no logical necessity to assume them to be derived from a single one.

If, as seems likely from the present results, the heat of activation *A* is unaffected by the length of the muscle, the heat can be written

$$H = A + ax,$$

where *x* cm. is the shortening and *a* the heat of shortening per cm. The heat of activation is apparently one-half to one-third the maximum heat under a small load. Taking the maximum shortening in a twitch as about  $l/3$ , where *l* is the length of the muscle, the maximum heat is then  $A + al/3$ . If *A* is one-half of this, it is equal to the heat of shortening one-third the length of the muscle; if *A* is one-third of it, it is equal to the heat of shortening one-sixth the length of the muscle. It seems that the heat of activation is rather less than the maximum heat of shortening. This gives an idea of its relative size and importance.

In this paper reference has been made only to the heat produced up to the end of contraction, not including that in relaxation and recovery. The recovery heat is a much slower phenomenon, taking thousands of times as long as the initial heat. There is no heat in relaxation, apart from that due to the dissipation of mechanical energy developed in contraction. This was shown in an earlier paper (Hill 1938, p. 168) and has been confirmed with much greater precision in recent work on the single twitch described in the following paper. Thus, apart from mechanical work

degraded into heat when the muscle relaxes under tension, the heat production in the muscle twitch comes entirely under the two headings, heat of activation and heat of shortening. There is never any sign of an endothermic process during contraction and relaxation.

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## The energetics of relaxation in a muscle twitch

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When a muscle relaxes under a load heat is produced which is equivalent, or nearly equivalent, to the mechanical energy which disappears. The appearance of this heat is simultaneous with the disappearance of the mechanical energy.

When a muscle is without load, or under zero tension, during the interval normally occupied by relaxation, there is no measureable heat after shortening is complete. This is true whatever the initial load, or the work done. If chemical changes occur during relaxation their net thermal effect is negligibly small.

The heat produced in an isometric contraction is of a very complex nature, involving internal shortening, the transfer of work from one part to another and the dissipation of mechanical energy as heat.

The nature of relaxation is discussed, and an analogy described with the process of 'cold-drawing' of a crystalline long-chain polymer.

By muscular 'relaxation' is meant the process by which a muscle returns, after contraction, to its initial length or tension. It occurs rapidly and must not be confused with 'recovery' which takes thousands of times longer. During contraction, unless it is forcibly stretched, a muscle shortens: even under so-called 'isometric' conditions its contractile elements shorten by stretching the elastic elements in series with them, indeed the form of the isometric contraction is largely determined by these internal readjustments of length (Hill 1938, p. 186; Katz 1939, p. 50). When relaxation sets in, the contractile elements cease progressively to be able to bear the load they raised earlier, or the tension they developed. They 'give', like a wire stressed beyond its elastic limit, and in 'giving' transform into

heat the mechanical potential energy of the load they previously lifted or the elastic energy of the structures they previously stretched. Figure 3 below shows how exactly the heat set free in relaxation coincides in time with the falling of the load.

A better analogy, perhaps, than a wire is that of a thread of some crystalline long-chain polymer, e.g. polythene or nylon. Such a thread, as extruded, in which the crystalline regions are originally unoriented, is drawn out, on stretching, to a state in which the crystalline regions are parallel: in this process of 'cold-drawing' a considerable amount of mechanical energy is degraded into heat. The thread, originally plastic, becomes reversibly elastic when fully drawn out (Bunn & Alcock 1945). If stimulated under a moderate load a skeletal muscle returns, again and again, to the same length. If the load is replaced by a very small one (of the order of 1% of what it could lift if stimulated) the muscle shortens passively and reversibly a small amount. Now stimulated, it shortens as usual but does not relax of itself to its initial length, but remains much shorter. If gently pulled out it returns to the length it had before under the very small load. From the plastic state remaining after contraction it returns, when gently pulled out but not otherwise, to the original reversibly elastic condition. In the pulling out process heat is produced.

Changes of double refraction occur in muscle with changes of length. Von Muralt (1932) showed that in isometric contraction of a frog's striated muscle there is a rapid decrease of double refraction at first when the contractile elements of the muscle must have been shortening internally. Bozler & Cottrell (1937) also found a considerable decrease of double refraction in the isometric contraction of a frog's sartorius under low initial tension (a condition favouring internal shortening, since the series elastic structures are much more compliant at a low tension), but in smooth muscles (the retractors of *Helix pomatia* and *Phascolosoma*) they found no change of double refraction under isometric conditions. They did find, however, a considerable increase of birefringence when a resting smooth muscle was stretched (which could be done without increasing its tension permanently), and concluded that changes of double refraction are not directly associated with changes of tension, or with activity, as such but are due to changes of length: at a greater length more molecules are oriented parallel to one another. These changes of double refraction in muscle on lengthening are suggestively similar to the changes of crystalline orientation in fibres of long-chain polymers on cold-drawing.

Relaxation in muscle has generally been regarded not as a passive but as an active process, and the considerable amount of heat produced during relaxation seemed to bear out this view. It appears, however, that this heat, or far the greater part of it, is due simply to mechanical energy dissipated when the load drops or the elastic extension vanishes. For isotonic tetanic contractions the matter has been fully discussed before (Hill 1938, p. 168) and experiments with the much more rapid equipment now available have confirmed, on single twitches, that the heat appears exactly at the same time as the work disappears. Whether the relaxation heat is precisely equal to the work dissipated cannot be established directly. It is certainly nearly equal to it, but there are technical difficulties in



establishing an exact equality. Relaxation under a load is very rapid and there is no reason to expect it to start at the same time, or to proceed at the same rate, in all fibres. Of two fibres in parallel the one which relaxed later would absorb most of the energy of a falling load. Of two regions of the same fibre in series, the greater share of the elastic energy of a muscle under tension would appear as heat in the region which relaxed earlier. The myothermic method requires that the heat should be uniformly distributed. If it is not, serious errors may result, particularly if small differences are being looked for. This complication, therefore, makes it very hard to measure the relaxation heat with the precision necessary to establish its equality to the work absorbed. All that can be said is that the two quantities are approximately equal and that no consistent differences appear. Even if the absorbed work is the direct cause of the relaxation heat they need not necessarily be exactly equal. When a sample of long-chain polymer, e.g. polythene, which is largely crystalline at ordinary temperature, is warmed up it absorbs far more heat than would correspond to any ordinary specific heat. This is due to the decrease of crystallinity, the greater randomization, with rising temperature (Raine, Richards & Ryder 1945). The heat content of the crystal, which is the more orderly arrangement, is less. On this analogy, since the muscle (as judged by its birefringence) has a more ordered molecular pattern at the greater length, we might expect the heat produced when a muscle relaxes under a load, or is pulled out after shortening under a very small load, to be slightly greater than the work. The difference, however, is bound to be very small, too small probably for accurate measurement.

The question whether there is any remainder when the work absorbed in relaxation is subtracted from the relaxation heat has a special relation to the problem of whether relaxation is an active or a passive process. If any of the chemical changes known or supposed to be associated with muscular activity, in particular the breakdown of creatine phosphoric acid and of adenosine triphosphate (ATP) and the formation of lactic acid, do not occur entirely in recovery, are they associated only with the phase of contraction, or are they connected also with relaxation? If the latter, it is probable that heat would appear as their accompaniment. Now it is possible to prevent any work from being transformed into heat in relaxation (if one may now so call it) by arranging the conditions of contraction so that the force on the muscle is zero at the time when relaxation would normally go on. If chemical changes are associated with relaxation when it visibly occurs under a load, it is unlikely that they would be completely absent during the same interval when a load was lacking. Experiments therefore, have been made to find out whether there is any heat production during the interval normally associated with relaxation, when the muscle is subjected to no load or tension during that interval.

The results were decisive, there is no such heat; or if there is any, it is not more than 1% or so of the heat previously liberated during shortening. The first method employed was to stimulate the muscle under a very small load, so small that visible relaxation either did not occur or occurred only to a minor extent. The muscle, on a thermopile, was pulled out gently to the greatest length at which it would remain under the very small load. A single shock was given, the muscle shortened considerably and remained short, and the heat was recorded photo-

graphically for 2 or 3 sec., covering the whole period of normal relaxation under a load. The muscle was then gently pulled out to its former length and the process was repeated. Several such records were made and their mean taken. It was now necessary to apply a correction for heat loss: no other analysis was necessary. For this purpose the muscle was heated electrically for 0.1 sec. by a high-frequency (100 kc./sec.) current which did not stimulate it and a record made for 10 or 15 sec. The rate of fall of the deflexion gave the coefficient of heat loss, which could be expressed as  $x\%$  of the deflexion per sec., where  $x$  might be from 3 to 7 depending on the size of the muscle.

With allowance for heat loss the deflexion representing the heat became approximately constant after the shortening phase of contraction was complete. The result, however, varied somewhat, sometimes there appeared to be a small positive heat production during and after the relaxation interval, sometimes a small negative one. The appearance resembled what one knew to be the effect of slight inequalities of heat production between different parts of the muscle (Hill 1938, p. 150). If the part of the muscle in contact with the thermopile contracted less vigorously than the part more distant from it, there would seem to be a delayed production of heat: and vice versa. The effect could be avoided by repeating the observations with the muscle reversed on the thermopile. The mean was taken of the records obtained with the thermopile in contact successively with the two opposite surfaces of the muscle, and the correction for heat loss was applied to the mean. When this was done the irregularities disappeared, confirming the impression that they were due to inequalities of heat production in the muscle. The result was that there is no measurable heat production during the whole interval normally occupied by relaxation.

This conclusion was reached under very special conditions, viz. in a contraction with very small initial load in which practically no work was done. It might conceivably be different in a contraction under more usual conditions, so it was thought desirable to investigate the matter further in muscle twitches starting under a moderate initial load and doing a substantial amount of work. The obvious suggestion was to use afterloaded isotonic twitches and to hold up the load when shortening was complete: the muscle could then 'relax' under zero load. The objection, however, to this was that even if the load did not fall back a state of tension would continue to exist in the muscle, and the elastic potential energy would be turned into heat in relaxation. It was necessary to devise some arrangement by which the load gradually diminished as contraction proceeded, becoming zero by the time that shortening was complete. For this purpose the ergometer was constructed which is shown in figure 1.

#### *Details of ergometer*

The muscle was connected as usual by a light chain  $P$  to the lever  $D$  revolving about the miniature ball-bearing  $L$ . When it was used isotonicly a weight was placed in a pan connected to the thread  $N$  which passed round the pulley  $M$ . When used with the ergometer, the weight merely balanced the lever and chain. The lever was connected by a thread  $C$  to the ergometer lever  $A$ , passing round

a pulley *B*. The point of the lever *A* rested on the top of the adjustable screw *H* in front of a millimetre scale *J* (which gives the scale of the drawing).

When the muscle shortened the pulley *B* revolved, raising the lever *A* and the weight and pan *G*. If the weight was not too great the muscle was able to lift it until *E* came vertically over *B* and the lever then tipped over, applying no further load to the muscle. If the weight was great enough the muscle could not lift it over the top and the lever fell back, the work done being absorbed by the muscle in relaxation. If the lever *A* was initially horizontal the weight was lifted a distance equal to *EB*. If it was not, the distance lifted was given by the initial position of the pointer on the millimetre scale. The work done was the product of the weight and the distance lifted.

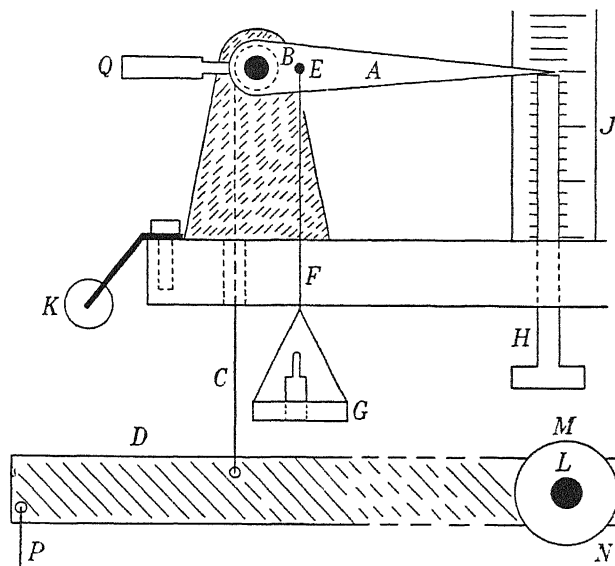


FIGURE 1. Ergometer. *A*, dural lever on 2 mm. shaft with sapphire pivot and 2 mm. ball race. On the shaft is pulley *B*, 4 mm. diameter, carrying silk thread *C* connected to isotonic lever *D*. At *E* a pin projects from *A* bearing, on a 1 mm. ball race, a small brass carriage (not shown) holding the thread *F* to the pan *G* containing weights. *H*, adjustable screw; *J*, millimetre scale; *K*, sponge rubber held on wire; *L*, ball bearing of isotonic lever; *M* pulley for thread *N* carrying weights; *P*, chain connected to muscle; *Q*, counterweight.

When the ergometer was pulled 'over' the energy of the load was absorbed by the lever hitting the sponge rubber *K* held at the end of a wire.

The load on the muscle follows a sine curve, its maximum being with the lever horizontal, zero with the lever vertical and beyond. By varying the load and the distance lifted optimum conditions can be found. Efficiencies up to 40 % were obtained.

The whole arrangement in figure 1 was mounted on a Palmer stand with vertical screw adjustment. The ergometer was carried on a separate adjustable rack attached to the stand. The initial tension of the muscle could be adjusted as desired.

With this ergometer the results shown in figures 2 and 3 were obtained. In figure 2 (frog's muscle) the load on the ergometer was finely adjusted to values

which the muscle just could and just could not lift 'over'. In *A*, with the work done during shortening not absorbed by the muscle in relaxation, there is no relaxation heat. In *B*, with the load falling back, there is a large and sudden heat production in relaxation. In figure 3 (toad's muscle) simultaneous records of heat and shortening are given for two contractions with loads well above and below the critical value. Records *B* show how sharply the relaxation heat coincides with the fall back of the load.

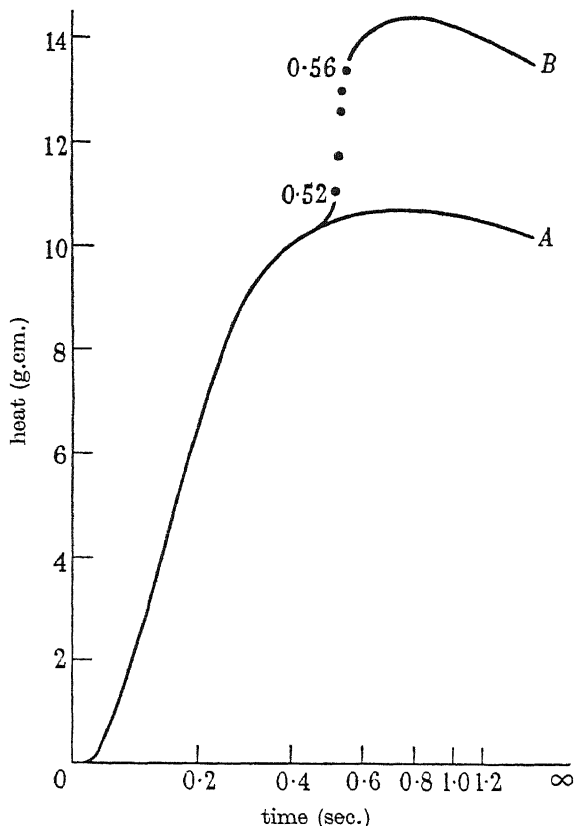


FIGURE 2. To show 'relaxation heat'. Two records superimposed of the heat production in two successive twitches of a frog's sartorius (113 mg.) at  $0^{\circ}\text{C}$ , using the ergometer shown in figure 1. Heat in mechanical units, time on exponential scale. *A*, 10.9 g. on ergometer just lifted 'over' 4.4 mm.: heat 10.7 g.cm., work 4.8 g.cm. *B*, 11.9 g. on ergometer just failed to be lifted 'over' and fell back, heat+work degraded into heat 14.3 g.cm. The five time spots on record *B* are from 0.52 to 0.56 sec.

When the load was tipped over the muscle 'relaxed' under zero tension. The same procedure was adopted as before. Records were made with the muscle both ways round on the thermopile, and the mean taken. A correction for heat loss was then applied. The result was the same, there is no significant heat after shortening is complete. With the ergometer the initial load could be adjusted as desired, and the work done could be varied from almost nothing up to two thirds of the heat: but such variation did not affect the result.

It has been suggested by Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen (1944, p. 391) that combination of ATP with myosin is the immediate cause of shortening and that relaxation and recharging occur at the expense of free energy derived from the splitting of the ATP. If chemical changes (apart from the very slow ones associated with recovery) do occur after shortening is complete they are not associated with any measurable liberation of heat. If ATP is broken down, or restored, during the interval normally associated with relaxation, its heat production, or absorption, is exactly compensated by that of some other process.

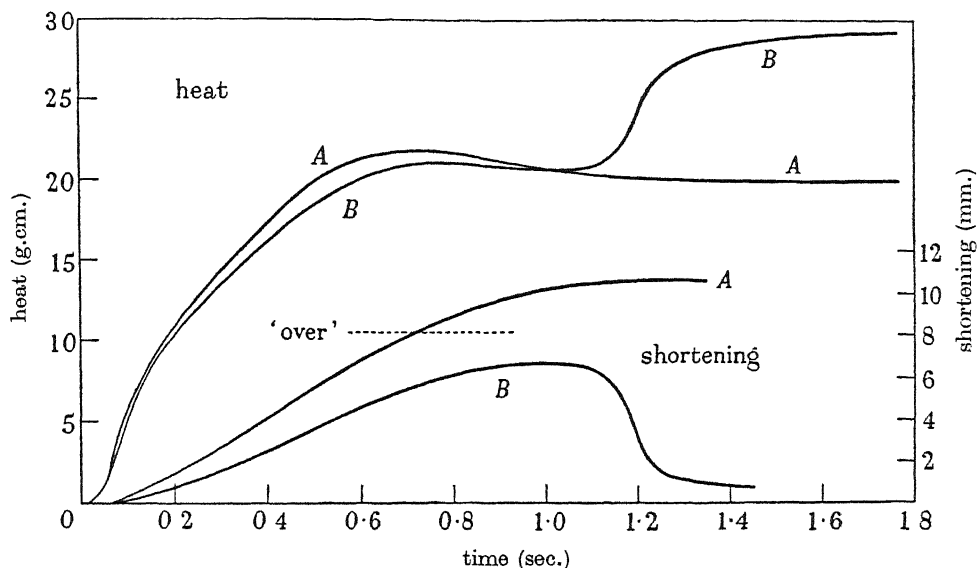


FIGURE 3. To show 'relaxation heat'. Records of the heat production and shortening in consecutive twitches of a pair of toad's semi-membranosi (155 mg.) at  $0^{\circ}\text{C}$  using the ergometer. *A*, 20.7 g. on ergometer lifted 'over' 4.9 mm. ( $\equiv$  8.1 mm. shortening): heat 21.9 g.cm., work 10.1 g.cm. *B*, 25.7 g. on ergometer not lifted 'over'. In *A*, the total shortening was 10.5 mm. and the efficiency was 32%. In *B*, the maximum shortening was 6.6 mm., the work done was 12 g.cm. and the efficiency was 36%, the work being dissipated as heat in the muscle as it relaxed.

Let us consider finally the very complex case of the relaxation heat in an isometric contraction. Many papers have been written about this: the main conclusion of them (e.g. Hartree 1931; Bozler 1936; Hill 1938, p. 187; Hartree & Hill 1928) was that the relaxation heat is equivalent to the elastic energy developed during contraction. While generally true this is not quite the whole story as the following considerations show.

In figure 4 curves *A* and *B* represent the heat and the tension developed in an isometric twitch of a pair of toad muscles. The contraction was a strong one, a force of about  $1\frac{1}{2}$  kg. per sq.cm. being developed. There is clearly no sharp division between the heats of contraction and relaxation: it would be quite arbitrary to take the moment of maximum tension as the dividing point. It is obvious, however, from the sudden upward turn of the heat curve after 1.5 sec., at the time when the

tension is falling most rapidly, that the disappearing elastic energy is providing a large part of the extra heat. On the other hand the heat is increasing rapidly at about 1 sec. when the tension is momentarily steady.

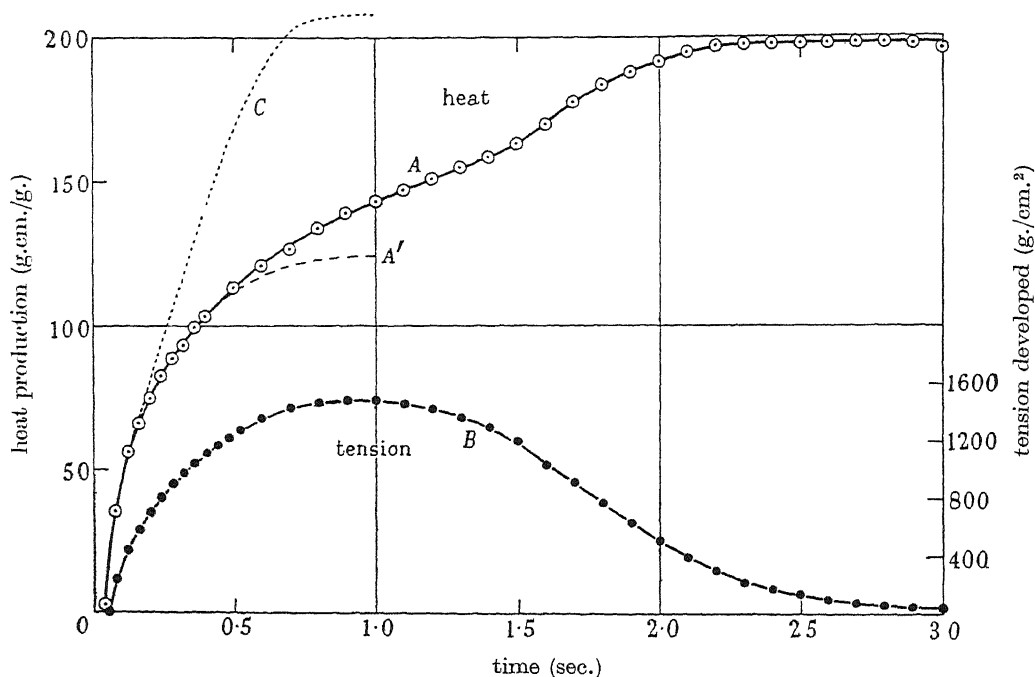


FIGURE 4. Heat *A* and tension *B* simultaneously recorded in twitch (mean of two) of two semi-membranosi (132 mg.) of toad at 0°C. Initial tension 6 g. (120 g./cm.<sup>2</sup>), length 25 mm. Heat analyzed at first in 40 msec. units, later in 100 msec. units, as shown by circles. *C*, heat production (analyzed) in isotonic twitch under 6 g. load (shortening, recorded but not shown, 10.4 mm.). *A'*, hypothetical form of *A* assuming no work degraded into heat.

According to the previous paper the heat production in a twitch is made up of two parts, the heat of activation and the heat of shortening. Curve *C* represents the heat in an isotonic shortening of 10.4 mm. In the isometric twitch the internal shortening of the contractile elements against the rising tension of the elastic ones cannot have been more than about 10%, say 2 or 3 mm., and must have been nearly complete by 0.8 sec.\* (see Hill 1938, p. 186; Katz 1939, p. 52). The heat of activation plus the heat of shortening cannot have been far from the broken line *A'* which has been somewhat arbitrarily drawn in. The excess of *A* over *A'*, starting as early as it does, cannot all be due to dissipated elastic energy: some of it must come from elsewhere.

We cannot assume that each of the several hundred fibres composing a muscle is of uniform cross-section and strength throughout its length. If one region of a fibre were stronger than another a stage would be reached in an isometric contraction at which the weaker region could no longer bear the tension developed by

\* For a frog's muscle at 0°C the internal shortening in a twitch would be nearly complete in 0.4 sec.: for a toad's muscle, with about half the value of *b*, 0.8 sec. is more appropriate.

the stronger region: it would 'give', allowing the stronger region to do work in stretching it. Such stretching is mainly an irreversible process and the work done is largely turned into heat (see Hill 1938, figure 14, p. 180). But when a muscle does work, it liberates extra energy equal to the work: if the work done by one region of a fibre is used in stretching another region, it will appear in the latter largely as heat, and the total heat will be greater. This is probably the explanation of the early divergence of  $A$  from  $A'$ .

The heat, therefore, in an isometric contraction is made up of four parts: (i) the heat of activation, (ii) the heat of shortening of the contractile elements in extending the elastic elements, (iii) the work done by the stronger regions of any fibre in extending the weaker regions, this work being turned largely into heat in the latter, and (iv) the elastic energy developed during contraction which is turned into heat in relaxation. None of these can be accurately assessed and it is a misfortune that so much work has been spent in studying the isometric contraction, with all its apparent simplicity and actual complexity. The reason for using the isometric contraction for myothermic work was, in the past, that shortening might bring regions of the muscle at a different temperature on to the thermopile and lead to serious and uncontrollable errors. The 'protected' thermopile (Hill 1937) used in the present work avoids those errors, and allows the simpler types of contraction to be properly investigated.

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# Work and heat in a muscle twitch

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In a single twitch, when a muscle shortens against a load, doing work, the heat produced is independent of the work done, provided the amount of shortening is kept constant. The total energy liberated may be expressed as  $(A + W + ax)$ , where  $A$  is the heat of activation,  $W$  is the work and  $ax$  is the heat of shortening. This relation is true not merely for the whole contraction but for any part of it.

The rate at which energy is liberated by a muscle during a twitch, in excess of the activation heat, is a decreasing linear function of the load  $P$ :  $dW/dt + a dx/dt = b(P_0 - P)$ . This relation is the basis of the characteristic equation connecting the speed of shortening to the load.

These relations are the same as were previously found for tetanic contractions, with similar constants.

The active state appears to be set up suddenly very soon after a shock.

The physical basis of these conclusions is discussed.

It was shown (Hill 1938) that when a muscle shortens in a tetanically maintained contraction it liberates extra energy in two forms, (i) as 'shortening heat', in amount proportional to the shortening, and (ii) as external mechanical work. The shortening heat per cm. of shortening was independent of the load, and therefore of the work done and the speed of shortening.

In the first paper of the present series it was shown that, apart from mechanical energy turned into heat in relaxation, the heat produced in a single twitch occurs in two forms, (1) heat of activation, which (as a first approximation at least) is independent of length, load, shortening or work, and (2) heat of shortening, proportional to the amount of shortening but, per unit of shortening, independent of its speed and therefore independent of load. In addition to these heats, energy is liberated in the form of mechanical work, depending on the load and the shortening. The first purpose of this paper is to show that, provided the amount of shortening is kept constant, the work can be varied over a wide range without any effect on the heat: or, more generally, that the total energy  $E$  set free in a muscle twitch can be expressed in the form:

$$E = A + W + ax,$$

where  $A$  is the heat of activation,  $W$  is the work done,  $x$  is the amount of shortening and  $a$  is a constant of the order of 350 g.cm. per cm. shortening, reckoned per sq. cm. of muscle cross-section.

In order to vary the work without changing the amount of shortening, the ergometer described in the preceding paper was used. With initial length constant, the load could be varied from the least weight required to hold the lever  $A$  down to the screw  $H$ , up to the greatest which the muscle would lift 'over'. Provided that the weight *was* lifted 'over' the muscle was then left free to shorten as much as it could. With frogs' muscles the total amount of shortening was practically



the same whatever the load on the ergometer. With toads' muscles the total shortening was rather less with the greater loads: the reason for this was that more time was taken in lifting the greater loads, so less time remained for subsequent shortening. Toads' (*Bufo bufo*) muscles shorten considerably more in a twitch than frogs', so the effect of the greater load was more apparent.

Since the ergometer lever was always tipped over, no load remained on the muscle after shortening and the heat, recorded photographically, contained no degraded mechanical work. A small correction was applied to the heat record to allow for heat loss but no other analysis was necessary. In order to make the results independent of possible progressive change in the muscle, observations were recorded at regular intervals and repeated in the reverse order, or otherwise distributed so as to eliminate change. In many cases the whole series was repeated with the muscle reversed on the thermopile, and mean values taken to avoid the effects of possible inequality between the inside and outside of the muscle. Details of two experiments on frogs' *sartorii* follow.

*Experiment 1.* Muscle 34 mm. long, 113 mg., 0°C. Single shocks (supermaximal) every 3 min. Ergometer provided initial load 0.86 g., afterloads 0, 8 and 10 g. Additional isotonic load 0.43 g., lifted 10 mm. Ergometer load lifted 4.05 mm., then 'over'. Muscle failed to lift 11.86 g. 'over', large relaxation heat recorded.

load on ergometer (g.)	0.86	8.86	10.86
heat (g.cm.)	10.1	—	—
	10.1	—	—
	—	10.3	—
	—	10.4	—
	10.1	—	—
	10.0	—	—
	—	10.2	—
	—	10.5	—
	10.5	—	—
	10.5	—	—
	—	10.5	—
	—	10.2	—
	10.3	—	—
	10.4	—	—
	—	—	10.7
mean heat	10.25	10.35	(10.7)
work (g.cm.)	0.8	4.0	4.8
efficiency (%)	7	28	31

*Experiment 2.* Muscle pair, 174 mg. Twitches in succession, load varied. 0°C.

work (g.cm.)	0.7	4.4	6.5	7.2	0.7	2.4	4.8
heat (g.cm.)	18.3	17.8	17.9	18.4	18.8	19.0	18.6
work (g.cm.)	7.2	2.4	0.7	0.7	6.8	3.5	0.7
heat (g.cm.)	19.2	19.9	19.9	20.6	21.7	19.9	20.6

In experiment 1 the work changed from 0.8 to 4.0 g.cm. without any significant change in the heat. The heats in experiment 2, if plotted successively, show a slight progressive increase in the response of the muscle, but no correlation at all between heat and work. Eight other experiments at 0°C on frog's muscle are

summarized in the following table. Each recorded number is the mean in a series and reverse with varying load, sometimes several times repeated, so as to avoid the effect of any progressive change in the muscle. In each experiment the heats are expressed as percentages of the heat with the smallest load. In order to make the results of different experiments comparable, the work is given as a percentage of the heat. The average value of the heat was 125 g.cm. (about  $3 \times 10^{-3}$  cal.) per g. of muscle.

work/heat (%)	6	12	16	19	22	25	27	27	30	32	35	39	45	45	47	mean
heat (%)	100	99	99	100	99	93	98	103	100	95	102	101	94	100	92	98

We see how the work can be varied from almost nothing to nearly half the heat, without any effect on the heat.

With toads' muscles the results were slightly complicated by the occurrence of rather less shortening after lifting greater loads. The following experiment is typical.

*Experiment 3.* Toad's sartorius, 75 mg., 32 mm. long, 0°C. 'Over' 3.9 mm. corresponded to 7 mm. shortening. Each result is the mean of four, in a series and reverse, repeated with the muscle turned round on the thermopile.

load lifted (g.)	1.74	5.74	10.74	15.74
heat (g.cm.)	10.15	10.1	9.7	9.35
work (g.cm.)	0.7	2.2	4.2	6.1
efficiency (%)	6	18	30	39
max. shortening (mm.)	14.1	13.6	12.9	11.5

If the differences of heat were due to differences of shortening they would correspond to a heat of shortening of 3.5 g.cm. per cm., or 160 per sq. cm. of muscle cross-section. This is smaller than the mean value found in the previous paper, over the main range of shortening, but a smaller value at the extreme limit of shortening was indicated by the results there given.

Other similar experiments showed the same result. With greater loads the heat was generally slightly less, but so was the total shortening, the difference requiring a reasonable allowance for the heat of shortening.

We may conclude therefore that when the work is varied by changing the load there is no corresponding variation of the heat, except in so far as the shortening also is changed. The equation:

$$E = A + W + ax$$

gives us three separate terms for the total energy; the heat of activation, the work and the heat of shortening. In general the amount of shortening will depend on the conditions of loading, so to that degree the second and third terms are not strictly independent. In an isotonic contraction under load  $P$  the equation reduces to the simple form:

$$E = A + (P + a) x.$$

These equations should hold, not only for the whole contraction but for any part of it; in the last one, for example,  $E$ ,  $A$  and  $x$  can all be regarded as functions of  $t$ , the time after the shock.

## DISCUSSION

The results of this paper confirm, on the single twitch, the conclusions reached in the earlier investigation (Hill 1938) of the tetanic contraction. They differ substantially, however, from those of previous papers. Hartree & Hill (1928*b*), for example, concluded that in a twitch the total energy set free is the same whether work is done or not. The chief reason for the disagreement is that they took the isometric twitch as the standard example of a contraction in which no work is done, and compared the total energy in a contraction in which external work was performed with the heat under isometric conditions. We know now (see the preceding paper) that the isometric contraction is a very complex affair, in which a considerable amount of work is actually done and transformed into heat both during and after contraction. Hartree & Hill were unaware of the shortening heat and their results on muscles allowed to shorten may have been affected by the errors\* which dogged the footsteps of all myothermic investigation before the 'protected' thermopile was introduced. Their results on tetanic contractions are similarly suspect. In fact, no conclusions for which isometric contractions were taken as standard, for comparison with contractions in which work was performed, can now be accepted as valid: while any earlier results of experiments in which shortening occurred ought now, before acceptance, to be checked with 'protected' thermopiles.

In an earlier paper (1928*a*), on the maximum work and mechanical efficiency, Hartree & Hill did not assume the isometric contraction as the base-line of zero work: their results are in principle valid, but the lowness of the maximum efficiency they obtained (26 %) is suspicious in view of the much higher values (up to 40 %) found later by Hill (1939*b*) and in the present work. Possibly the classical error due to differences of temperature along a muscle shortening on to an 'unprotected' thermopile may have made their heat readings too high. Wyman's (1926) results on the relation of work and heat in tortoise muscle should now be repeated in view of present knowledge and with the much better instruments now available.

Most of the earlier myothermic observations did no more than record the maximum deflexion, giving the total heat. As we now know, the heat production is a very complex business and present methods, allowing a more or less undistorted

\* These errors were due to differences of temperature along the length of the muscle. Even at rest, and particularly during recovery from previous activity, a muscle produces enough heat to keep its temperature measurably above that of its surroundings. The excess temperature is inversely proportional to the thermal conductivity of the environment, so that the part of the muscle lying on the thermopile is bound to have a lower temperature than the part beyond it surrounded only by poorly conducting gas. If therefore a muscle is allowed to shorten, parts of it previously off the thermopile come on to it and the rise of temperature recorded is due not only to heat production but to a warmer region of the muscle making contact with the thermo-junctions. This is avoided by 'protecting' the active region of the thermopile by a similar 'dummy' region beyond it, long enough to ensure that any part of the muscle coming on to the active thermo-junctions has been losing heat previously at precisely the same rate as the part already on them. The protected thermopile has been described in earlier papers (Hill 1937, 1938, 1939*a*). Its heavy metal frame, together with good thermostat control, ensures that no significant differences of temperature exist along the thermopile itself.

picture of its whole course to be recorded photographically, give much greater facility for understanding its nature. In the past a laborious numerical analysis was required before the time-course of the heat was revealed, and this could scarcely be applied as a routine in every kind of measurement.\* Consequently, much was missed which is now evident. Some of the results of older experiments in which shortening occurred (including those of Fenn (1923, 1924); Azuma (1924); Hartree (1925, 1928); Hill (1930); Feng (1932)) can now be interpreted in the light of present knowledge. Others ought to be repeated with present technique.

In the experiments (Hill 1938) on tetanic contractions it was found that the rate of extra energy liberation, over and above the 'maintenance heat', is a linear function of the load:

$$(P + a) \, dx/dt = b(P_0 - P),$$

where  $P_0$  is the isometric tension. This led to the characteristic equation:

$$(P + a) (v + b) = (P_0 + a) b,$$

relating the speed of shortening to the load. The constant  $a$  was the same whether derived from thermal measurements or from purely mechanical ones. The accuracy of the characteristic equation was examined in greater detail by Katz (1939) and has recently been confirmed by Wilkie (1949) for the contraction of human arm muscles.

The accuracy of the equation  $(P + a) \, dx/dt = b(P_0 - P)$  has been tested against the results of the present experiments and confirmed. The true value of  $P_0$  cannot be determined directly for a single twitch: relaxation sets in before the contractile elements of the muscle have had time to complete their shortening against the increasing tension of the series elastic elements (see Hill 1938, figure 15). If, however, we plot the observed values of  $(P + a) \, dx/dt$  against  $P$  in a series of isotonic contractions under different loads we obtain a straight line, the extrapolation of which back to the axis should give the true value of  $P_0$ . The fact that a linear relation is obtained confirms the equation. The procedure is illustrated by the following experiment.

*Experiment 4.* Pair of toad's semi-membranosi, 159 mg., 2.7 cm. long, 0°C. Single shocks. Initial load 6.0 g., afterloads 0.0, 15.6, 37.2 and 63.2 g. Heat and shortening simultaneously recorded, and heat of shortening,  $a = 32$  g.cm. per cm., calculated as described in a preceding paper. The curves of shortening are given in figure 1. They are linear over a considerable extent, the velocities being as follows:

load $P$ (g.)	6	21.6	43.2	69.2
velocity $dx/dt$ (cm./sec.)	1.8	1.02	0.50	0.17
$P + a$	38	53.6	75	101
$(P + a) \, dx/dt$	68	54.5	37.5	17

Adding  $a$  to  $P$  the values of  $(P + a) \, dx/dt$  are calculated in the last row. These are plotted in figure 2 (upper line). The linear relation shown has a slope  $b = 0.82$  and extrapolated to the horizontal axis gives  $P_0 = 90$ .

\* W. Hartree once estimated that in his analyses of muscle heat he had written down between  $10^7$  and  $10^8$  figures.

Similar calculations were applied to two other experiments. The lower line in figure 2 gives the relation for a pair of rather smaller toad muscles (116 mg., 25.5 mm.), the results being  $a = 15.5$ ,  $P_0 = 60$ ,  $b = 0.54$ . Another pair of toad muscles (69 mg., 24 mm.) gave  $a = 7.9$ ,  $P_0 = 23$ ,  $b = 0.66$ . The mean value of  $b$ , reckoned not in cm./sec. (as above) but in terms of muscle length per sec., was 0.26, while the mean value of  $a/P_0$  was 0.32. The mean values given (Hill 1938) by tetanic contractions of frog muscles at 0°C were,  $b = 0.33$ ,  $a/P_0 = 0.26$ . The

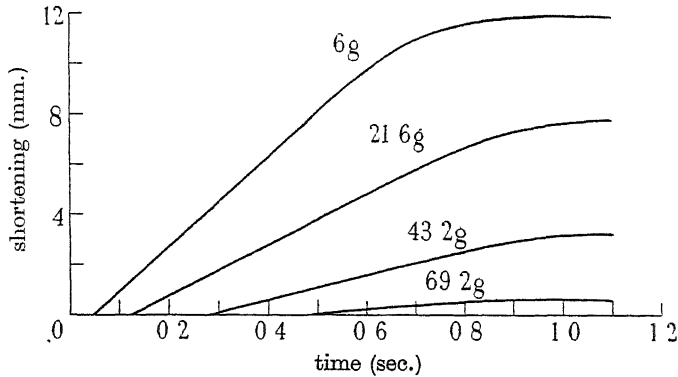


FIGURE 1. Isotonic contractions at 0°C, recorded photoelectrically, of pair of semi-membranous muscles of toad. 6 g. initial load, total loads as shown. For details see text.

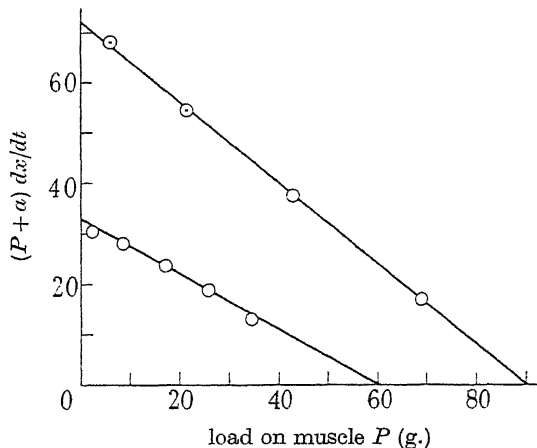


FIGURE 2.  $(P+a) dx/dt$  plotted against  $P$ .  $P$ , load (g.);  $x$ , shortening (cm.);  $ax$  heat of shortening (g.cm.);  $t$  time. Upper line for the experiment of figure 1. See text.

number of the present experiments is too small for accurate mean values, but it is clear that  $b$  and  $a/P_0$  are of the same order of size, the slower toad muscles having a rather smaller value of  $b$ .

It had been expected that  $b$  (expressed as a fraction of muscle length per sec.) would be about one-half as great in toad muscles as in frog muscles, in proportion to their relative speeds. It is clearly more than one-half. The reason may be as follows. Toad muscles can shorten considerably more than frog muscles, and  $b$

ought really to be expressed as a fraction of total possible shortening rather than of total length, per sec. Reckoned in this way  $b$  is probably about half as great as for frog muscle.

The physical basis of the fact that the rate of total energy liberation,  $(P + a) dx/dt$ , in excess of the heat of activation, is a linear function of the load, was discussed in the 1938 paper (p. 164). No new evidence of the nature of this simple but mysterious relation has appeared since: nor indeed has any new light been shed on the physical nature of the heat of shortening. It is very satisfactory, at any rate, that the present results with the single twitch agree so exactly with the earlier ones on the tetanically maintained contraction. The question naturally arises whether, or to what extent, the three quantities  $A$ ,  $W$  and  $ax$  which make up the total energy in a twitch are independent of one another, and whether they are derived from the same, or different, physical or chemical changes. That  $A$  is independent of the other two seems most probable: but the work  $W$  and the heat of shortening  $ax$  are connected with one another and the load by the relation we have been discussing, which can be integrated in the form  $W + ax = \int (P_0 - P) dt$ , so

they cannot be regarded as really independent. In fact, the load determines the rate and the total amount of shortening, and the work is determined by the load and the shortening. It is scarcely pertinent, therefore, to inquire whether work and heat of shortening are derived from independent physical or chemical changes.

If  $a$  could be regarded as a constant internal resistance to shortening the matter would be simpler, for then the shortening heat  $ax$  would be equivalent to the work done in overcoming this resistance and the whole of the energy set free in excess of the activation heat could be treated as work. An objection to this is that there should then be a difference  $2a$  between the loads (i) at which a muscle just shortens, and (ii) at which it just lengthens, and Katz's (1939) results show that the difference, if it exists, is much less than  $2a$ . This objection is avoided if a muscle fibre is regarded not as a single contractile element with a parallel constant resistance, but as a large number of such elements in series. At the full isometric tension the stronger of these might be on the point of shortening and stretching the weaker: a small decrease of tension would allow the former to shorten, a small increase of tension would stretch the latter, and the difference of tension between shortening and lengthening would be small. We cannot attribute the heat of shortening to a viscous resistance, for it is independent of the velocity of shortening. Whether  $ax$  changes sign when  $x$  changes sign (lengthening instead of shortening) is not certain: frogs' muscles do not stand stretching well enough to give decisive results (Hill 1938, p. 179). Possibly the jaw-muscles of dog-fish which stand stretching well (Levin & Wyman 1927, p. 224) would allow a decision to be reached. It may still be possible, when we come down to its molecular basis, to regard  $a$  as a constant internal resistance to shortening, so that  $(P + a) dx/dt$  would be the total rate at which work was being done. We should then have to think of a system in which the rate of doing work is a decreasing linear function of the load. No simple mechanical or electrical arrangement has this property and without some clearer idea of the general nature of the muscular machine it seems

useless to speculate further. Any model, however, of muscle must conform to the general pattern of these relations.

It has been a frequent astonishment in this work to find how accurately and consistently certain simple relations occurred, in some cases over a wide range. Toad muscles, for example, shorten by very considerable amounts, and one had expected that the heat of shortening per cm. would change as the muscle shortened. It might for example, have increased in proportion as the muscle got thicker, in which case the element of shortening heat would not be proportional to  $-\delta l$ , where  $l$  is length, but to  $-\delta l/l$ . To test this,  $\Delta H$  between two heats was plotted, not against  $-\Delta l$  as in the first paper of this series, to give the heat of shortening, but against  $-\Delta \log l$ . No regularity, however, was found. The heat of shortening appears to be proportional to the shortening itself over a wide range. Again, with a shock applied at the middle of a muscle with anodes near its ends, the form of the isotonic twitch, recorded without significant friction, is linear over a large part of its course as shown in figure 1: had it not been for this fortunate simplicity the argument relating speed of shortening to load would have been very complicated and no simple relation could have been established. The relatively small variation of the heat of shortening from muscle to muscle gave confidence that one was dealing with a real thing. The complete absence of relaxation heat when the muscle was under zero tension, and the fact that the amount of work done had no effect on the heat provided that shortening was constant, were other examples of simple regularities. They encourage one to believe that when the mechanism of muscular contraction is discovered it will be found to be of a rather simple nature.

One final regularity should be noticed, viz. the way in which all the relations here established appear consistently in the twitch from the earliest moment at which they can be examined. There is no sign of a gradual building up of a state of activity. No doubt that building up does take time, but the time taken seems to be very short. When a slower development of activity appears to occur it is found to depend on such things as propagation of contraction away from the stimulating electrodes, or on internal shortening by contractile elements against the rising tension of elastic elements. Gasser & Hill (1924) concluded from experiments on quick stretches that 'the fundamental mechanical response' attains its maximum intensity quite early in the contraction. Brown's experiments (1934, 1936) support the view that the greatest activity in a twitch occurs very soon after stimulation. The 'activation heat', which is probably a sign of the process by which the muscle is set ready to shorten or develop a force, begins very soon after a stimulus, with its maximum rate at the start; details will be given in a later paper. As soon as the whole muscle is excited it shortens at the full rate corresponding to the load. We may, in fact, regard the active state as being established suddenly soon after the shock. It is maintained during a tetanus; in a twitch it disappears, apparently without any accompanying heat, in the process known as relaxation.

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## Myothermic methods

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[Plate 8]

The recent development of galvanometers has led to substantial improvements in the technique of recording and analyzing the heat production of muscle, particularly in facilitating the correlation in time and magnitude between thermal and mechanical events. The heat changes can be resolved in time to within a few milliseconds; the changes of length or tension, recorded simultaneously, are practically unaffected by friction or inertia. The physical properties of the instruments, and numerical methods of analysis, are discussed.

## INTRODUCTION

The rise of temperature in a muscle twitch is small, being of the order of  $3 \times 10^{-3}^{\circ}\text{C}$ . To obtain sufficient accuracy it is necessary to record this to a few parts per thousand, say to  $10^{-5}^{\circ}\text{C}$ . But the heat production of muscle is a complicated process, and only a limited amount is learnt by simply measuring its total quantity; for most purposes it is necessary to record its course as a function of time. A single twitch, however, is a very rapid affair. Even at  $0^{\circ}\text{C}$  the heat in the twitch of a frogs' skeletal muscle begins to appear about 10 msec. after the stimulus, and is complete



in about 0.4 sec. High speed, therefore, as well as sensitivity, is required in the recording instruments.

The current sensitivity of a galvanometer is proportional to  $HT^2/a$  (Hill 1948*a*), where  $H$  is its magnetic field,  $T$  its period and  $a$  the width of its coil. Making  $H$  as large and  $a$  as small as practicable the sensitivity is proportional to the square of the period. A balance, therefore, must be struck between speed and sensitivity. The galvanometers available in 1938 were far slower than the thermopiles. Improvements, however, of the last few years (Downing 1948, Hill 1948 *a, b, c*) have made it possible, with adequate sensitivity for a single twitch, to follow the changing current from a thermopile with a time lag as small as  $1\frac{1}{2}$  to 4 msec. (see figure 6*c*). For many purposes the present speed of galvanometer and thermopile is so high that further analysis of the records is unnecessary; for some, however, it is still required. The rapidity of the galvanometer, moreover, has led to a better understanding of the properties of the thermopile, and so to simpler and more accurate methods of analysis when that is needed.

The galvanometer has been fully described elsewhere. Its deflexion is amplified photoelectrically 100 to 200 times and recorded on one beam of a double-beam cathode-ray tube. The second beam has been used simultaneously for the mechanical response (see figure 6*a*). Details of the display, timing and recording are given below.

The methods here discussed were developed primarily for the investigation, described in the following paper, of the rapid early heat production in a twitch. That required very high speed and sensitivity. The improved technique made possible the other investigations recently described (Hill 1949 *a, b, c*).

## 1. THERMOPILE

No reason has been found to modify the conclusion (Hill 1938, p. 157) that of all heat-measuring instruments practically available the 'protected' thermopile (Hill 1937, 1938) is the best suited to this work. The last thermopile constructed by Mr A. C. Downing before the war, referred to as P6, is quicker than had been anticipated: it is so thin that an instantaneous rise of temperature of a pair of muscles on it would give half the full thermo-e.m.f. in less than 1 msec., and delay in the arrival of heat at the recording junctions is due almost as much to the layer of connective tissue and water between muscle fibres and thermopile as to the thermopile itself. Any further improvement, therefore, in the direction of thinness would give rather little advantage. The requirement of a very high degree of insulation, to protect the recording galvanometer from electrical disturbances, is met by the mica insulation; this, with the bakelite varnish employed, provides a resistance between muscle and thermopile, after soaking in Ringer's solution for many hours, of 100 to 1000 megohms. Another thermopile, P7, constructed in 1945, has similar properties.

The only alteration necessary to the thermopile was to fix two stimulating electrodes, one on each side (i.e. one for each muscle), just over the middle of the region in which the heat is measured. The velocity of propagation of contraction

along a frog's muscle fibre at  $0^{\circ}\text{C}$  is about 30 to 40 cm./sec., so conduction over even a few millimetres involves a delay of a good many milliseconds. If the stimulating electrodes are distant from the thermopile, the latter cannot record any heat until contraction has been propagated to it and the advantage of high speed in the recording instruments is obscured. By placing the stimulating cathodes, one for each muscle, immediately over the region in which the heat is recorded, the delay is diminished. The two ordinary electrodes, one at each end, were used as anodes, so that the current from either cathode spread out both ways. The anodes were earthed; this helped to avoid disturbance of the galvanometer by the stimulus.

Details of thermopile P 6 are as follows. It has thirty couples of palladium-gold and iron, its resistance is  $17.1\ \Omega$ , it gives  $1363\ \mu\text{V}/1^{\circ}\text{C}$ , it is about  $40\ \mu$  thick. Its end electrodes (anodes) are of platinum wire rolled to the thickness of the thermopile and sealed to the thermopile by bakelite varnish; the middle electrodes (cathodes) are thin and flexible, being adjustable to make light contact with the outside of the muscles resting on the thermopile. The total length of the active region of the thermopile is 13.2 mm. There is a 'protecting' length of 1.8 mm. at the pelvic end, of 6.4 mm. at the tibial end (see Hill 1937, figure 4; 1938, figure 2). The muscles being placed so that their pelvic ends are 0.5 mm. beyond the electrode, the 6.4 mm. at the tibial end 'protects' the active elements of the thermopile for shortenings as great as 30 %. Another terminal of the thermopile is provided, making contact with only twenty-six couples instead of thirty, so permitting a further 2.4 mm. of 'protected' shortening, i.e. a total of 40 %. The twenty-six couples have a resistance of  $14.9\ \Omega$  and give  $1176\ \mu\text{V}/1^{\circ}\text{C}$ . For some of the experiments on toad muscles described in preceding papers (Hill 1949 *a*, *b*, *c*) in which 50 % shortening occurred, the active region of the thermopile was made still shorter.

In spite of the high speed with which the thermopile begins to record the rise of temperature of a muscle lying on it, there is bound to be considerable delay in reaching its full value. The final stages depend on the slow conduction of heat from the outer region of the muscle. Even with the thermopile so thin as to have a heat capacity equal to only about  $10\ \mu$  of muscle on each side, the attainment of the full rise of temperature by the junctions may take several hundred milliseconds. Moreover, when heat is produced by living fibres the delay due to the conduction of heat is made greater by a layer of connective tissue around the muscle and a film of fluid lying between the connective tissue and the mica. It is not possible to determine the thickness of these exactly, but connective tissue and Ringer's solution together appear to be about  $6\ \mu$  thick, which must be added to the  $10\ \mu$  equivalent to the thermopile on each side. Electrical heating of the muscle on the thermopile, as previously adopted, can reveal the constants of the instrument itself, but since inert conducting material is warmed to about the same extent as active muscle fibres, the thickness of the former must be allowed for independently. The 'heating controls' used in the analysis (see below) have taken approximate account of the heat capacity of the inert layer between active fibres and thermopile.

## 2. THE THERMAL CONDUCTIVITY OF MUSCLE

The flow of heat from muscle to thermopile depends chiefly on  $K$ , the thermal conductivity of muscle. From the thermal conductivities of its chief constituents, protein and water, the value of  $K$  was estimated (Hill 1937, p. 121) as  $1.24 \times 10^{-3}$  for frog's muscle containing 20 % of solids. By the kindness of Dr Ezer Griffiths, F.R.S., an absolute determination has been made at the National Physical Laboratory of the thermal conductivity of lean horse meat cut approximately parallel to the fibres; the value is  $1.1 \times 10^{-3}$  cal./cm.<sup>2</sup>  $\times$  sec. for 1 cm. thickness and 1° C difference of temperature. The hot-face temperature was 35° C, the cold-face 25° C. Over this range the thermal conductivity of water is  $1.5 \times 10^{-3}$ . The density of the meat was stated to be approximately 1.1 g./cm.<sup>3</sup>

In frog's muscle (Hill 1931, p. 277), for each 1 % of solid it contains the density is increased by 0.00286 as compared with Ringer's solution. Taking the latter as having a density at 18° C of 1.005, the density of a frog's muscle containing 20 % of solid would be 1.062. A density of 1.1 would correspond to about 33 % of solid; this seems unduly high and 25 % is more probable for horse muscle, which would correspond to a density of 1.077. If we interpolate linearly between 1.077 and 1.000 for frog's muscle containing 20 % of solid, with a density therefore of 1.062, the value of  $K$  for frog's muscle would be  $1.18 \times 10^{-3}$ . This is close to the  $1.24 \times 10^{-3}$  previously assumed.

In the equations it is not  $K$  but  $k = K/\rho c$  which occurs,  $\rho$  being density and  $c$  specific heat. For frog's muscle containing 20 % of solid,  $\rho = 1.062$  and  $c = 0.864$ . Hence for frog's muscle containing 20 % of solid  $k = K/\rho c = 1.29 \times 10^{-3}$ . The previous value was  $1.35 \times 10^{-3}$ . At 0° C,  $k$  must be somewhat lower, and for convenience the value  $1.25 \times 10^{-3}$  has been assumed in the calculations referred to below. If a muscle contained more solid its  $K$  and  $c$  would be less and its  $\rho$  greater; these changes balance each other approximately, and  $k = K/\rho c$  is nearly independent of the solid content.

## 3. HEAT FLOW FROM MUSCLE TO THERMOPILE

The equations for the flow of heat from muscle to thermopile were deduced in previous papers (Hill 1937, 1938). The thermopile is regarded as a sheet of thickness  $2b$  cm. with a muscle of thickness  $a$  cm. on each side. The heat flow is supposed to be perpendicular to the sheet; heat loss sideways along the thermopile (or outwards into the air) is slow and can be allowed for separately. Let  $K$  be the thermal conductivity of muscle,  $c$  its specific heat and  $\rho$  its density; put  $k = K/\rho c$ . For simplicity it is assumed that  $k$  is the same for thermopile as for muscle; actually it is somewhat greater, but no important error is introduced, the heat capacity of a very thin thermopile and the flow of heat in the muscles on it being the dominating factors; the net effect of assuming  $k$  to be the same for thermopile and muscle is to make the calculated speed of response of the thermopile slightly less than the actual speed.

If we suppose that the muscles are of infinite thickness and that at time zero their temperature is suddenly raised by  $y_0^\circ \text{C}$ , the temperature of the middle of the thermopile at any subsequent time  $t$  is

$$y/y_0 = 1 - \operatorname{erf}[b/2\sqrt{(kt)}], \quad (1)$$

where  $\operatorname{erf}(h)$  represents the probability integral  $\frac{2}{\sqrt{\pi}} \int_0^h e^{-x^2} dx$ . For the earlier stages of contraction, up to about 0.5 sec. for a muscle of ordinary size, equation (1) is sufficient. For longer times it is necessary to take account of the finite thickness of the muscle. Putting  $\gamma = a/b$  and writing  $x$  for  $b/2\sqrt{(kt)}$ , the temperature of the middle of the thermopile is

$$y/y_0 = 1 - \operatorname{erf}(x) + [\operatorname{erf}(1+2\gamma)x - \operatorname{erf}(3+2\gamma)x] + [\operatorname{erf}(3+4\gamma)x - \operatorname{erf}(5+4\gamma)x] + \dots \quad (2)$$

It is interesting to calculate approximately how rapidly the parts of the muscles distant from the thermopile affect its temperature. Imagine a layer of muscle, including the thermopile, of total thickness  $b'$  on each side, to be instantly heated and then allowed to cool down by conduction to an outer unheated region of infinite thickness and the same conductivity. From equation (1) the temperature of the thermopile, originally  $y_0$ , falls according to the equation  $y/y_0 = \operatorname{erf}[b'/2\sqrt{(kt)}]$ . Let us calculate how long it takes to fall 5% for various thicknesses of the heated layer. We put  $\operatorname{erf}[b'/2\sqrt{(kt)}] = 0.95$ , for which (from tables)  $b'/2\sqrt{(kt)} = 1.386$ . Putting  $k = 1.25 \times 10^{-3}$ ,  $b'/\sqrt{t} = 9.78 \times 10^{-2}$  and we calculate

$b' (\mu)$	50	100	150	200	250
$t$ (msec.)	2.62	10.5	23.5	42	65

Thus, within 42 msec. the reading is 5% affected by the heat produced by fibres as far away as 200  $\mu$  and beyond, within 10 msec. by the layer of fibres next to those in contact with the thermopile (say 100  $\mu$  away). If we take not 5% but 25% as the influence to be exerted by outer fibres, we put  $\operatorname{erf}[b'/2\sqrt{(kt)}] = 0.75$ , for which  $b'/2\sqrt{(kt)} = 0.814$ . Then we get  $b'/\sqrt{t} = 5.76 \times 10^{-2}$  and

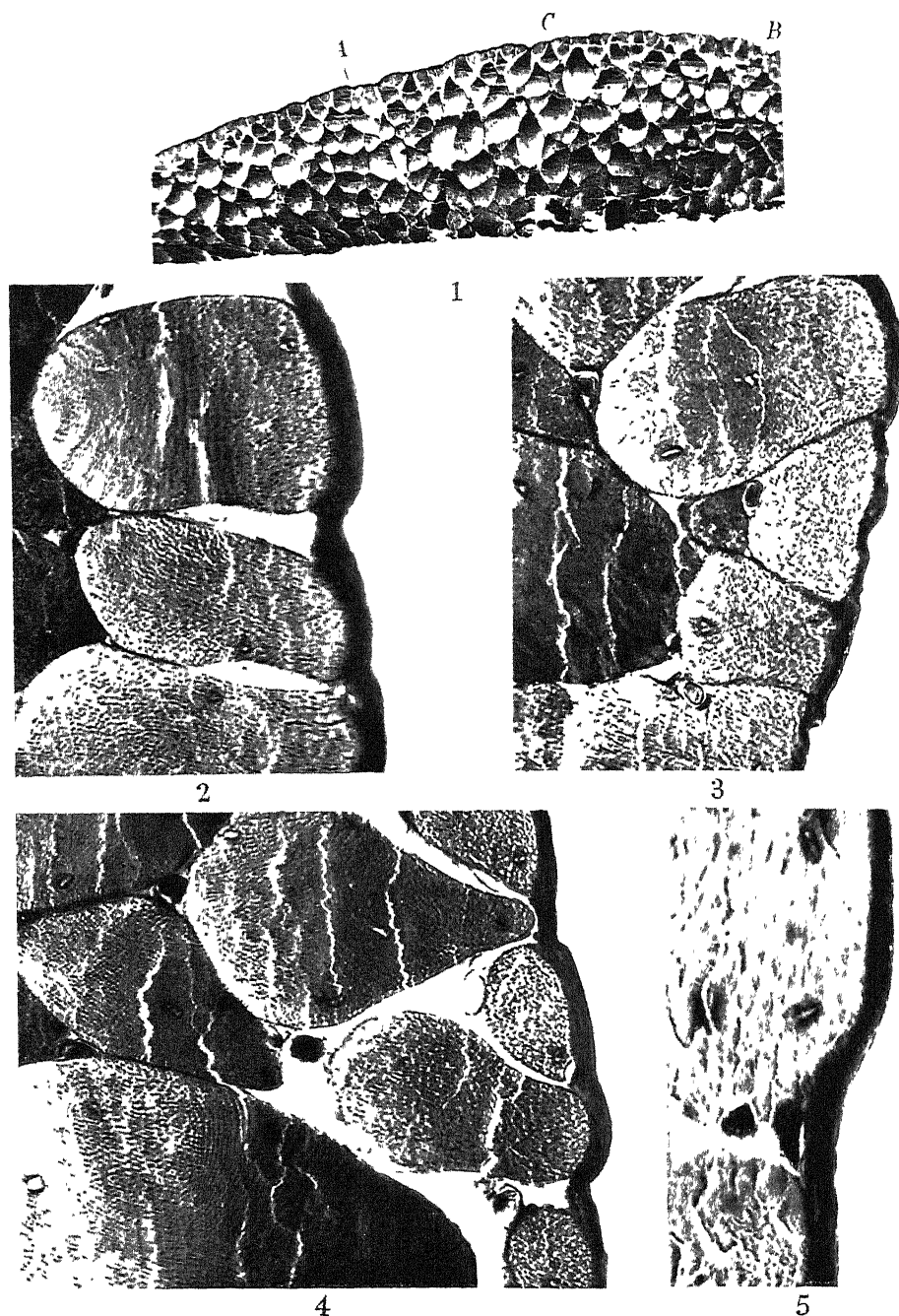
$b' (\mu)$	50	100	150	200	250
$t$ (msec.)	7.52	30.2	67.7	121	188

Thus the reading is largely affected within 30 msec. by what has happened in the layer of fibres immediately outside those in contact with the thermopile (say 100  $\mu$  away).

The general conclusion of this is that the layer of fibres immediately next to the thermopile is chiefly responsible for the earliest deflexion recorded, the more distant fibres contributing more at later times. For a true picture, therefore, of the course of the heat production, it is essential that the fibres at the surface should be in good condition and that the muscle should contract uniformly all over. Otherwise the only valid reading is the average obtained after 1 or 2 sec. when the heat has had time to be redistributed.

We have assumed hitherto that the rise of temperature is instantaneous. This condition is not realizable experimentally; the shortest practical interval of heating





Photomicrograms of the cross-section of a frog's sartorius muscle. Magnification figure 1,  $\times 41$ ; figures 2, 3 and 4,  $\times 500$ ; figure 5,  $\times 1000$ . To show the membrane on the 'outside' of the muscle (top in figure 1). Figures 2, 3 and 4 were taken from the regions A, B and C in figure 1; figure 5 from another muscle. Fixed in Boun's fluid, cleared in cedar-wood oil, embedded in paraffin,  $10\mu$  sections, stained in haematoxylin and van Gieson.

by a high-frequency current is a few milliseconds, which is a long time by the standards of the early heat flow into the thermopile. To compare theory and experiment it is necessary, by numerical integration from equation (1), to calculate the rise of temperature during and after a finite interval of heating. The observed deflexion for that duration of heating is analyzed numerically by the galvanometer deflexion to a constant current, to give the temperature of the thermopile as a function of time. The comparison of 'calculated' and 'observed' allows us to determine the constant factor  $b^2/k$  coming into all the equations. Assuming  $k = 1.25 \times 10^{-3}$ ,  $b$  is found. For thermopile P 6 the value of  $b$  so obtained is about  $10 \mu$ , that is to say, the whole thermopile has a heat capacity equal to about  $20 \mu$  of muscle. This is better than originally estimated. The thickness of thermopile P 6, as measured with a micrometer, is about  $40 \mu$ , about  $15 \mu$  of wire and  $2 \times 12\frac{1}{2} \mu$  of mica. About two-thirds of the space inside the mica is occupied by wire, so that the average value of  $\rho c$  (for mica 0.63, for metal 0.85) would be  $(25 \times 0.63 + 10 \times 0.85)/40 = 0.605$ . For frog's muscle  $\rho c$  is about 0.92, so that  $40 \mu$  of thermopile should be equivalent in heat capacity to  $40 \times 0.605/0.92 = 26.3 \mu$  of muscle. The fact that the heat capacity is only about three-quarters of the value so calculated may be due partly to the mica and wire being thinner than allowed for, partly to their thermal conductivity being higher than that of muscle. It is satisfactory that the speed of the thermopile (being inversely proportional to  $b^2$ ) is nearly twice as great as expected.

The experimental verification of equation (1) and the direct determination of  $b$  were made possible by the high speed of the new galvanometers. Several thermopiles were examined in this way. For P 7 the value of  $b$  was about  $10 \mu$ , for P 5 about  $11\frac{1}{2} \mu$ , for some of the older protected thermopiles substantially more.

#### 4. THE EFFECT OF CONNECTIVE TISSUE AND FLUID BETWEEN MUSCLE AND THERMOPILE

In a 'heating control' all the electrically conducting material present, muscle, Ringer's solution, fascia, connective tissue, etc., lying between the electrodes is nearly uniformly heated. When the muscle contracts, however, only the fibres produce heat, the inert material between them and the thermopile providing a heat capacity which, added to that of the thermopile, considerably slows the deflexion. If a heating control could be made, which is experimentally impossible, for the contractile material only, it would rise considerably more slowly. The consequence of carrying out an analysis with a heating control which rises too rapidly is to give a false result which is smaller, and occurs later, than it should.

The muscles used in several of the present experiments were afterwards fixed and, through the kindness of Professor J. Z. Young, sections of them were stained and prepared for photomicrography by Mr J. Armstrong of the Department of Anatomy at University College. Figure 1, plate 8, at a magnification of  $41 \times$ , shows the full thickness (0.64 mm.) and 2.1 mm. of the width of a frog's sartorius. The 'outside', i.e. the surface next the skin, is above, the 'inside' below. Figures 2, 3 and 4,

plate 8, at a magnification of  $500\times$ , are taken from the regions marked *A*, *B* and *C* respectively in figure 1. Figure 5, plate 8, is from another muscle at  $1000\times$ .

Figure 1 shows that around the 'outside' of the muscle a complete membrane exists which, in figures 2, 3 and 4, varied in thickness from about 4 to  $8\mu$ . Nothing comparable is seen on the 'inside'. In figure 5, on another muscle at a higher magnification, the thickness of the membrane averaged  $3.8\mu$ . It varies substantially from muscle to muscle and from region to region of the same muscle. In the living state it may be somewhat thicker, but no considerable shrinkage took place, during fixing, in the muscles as a whole.

Figure 1 shows one obvious peculiarity, which is found (though not always so strikingly) in other muscles, viz. that the fibres on the 'outside' tend to be smaller than in the middle. Since, in the usual arrangement using a pair of muscles, the 'outside' fibres are the ones which first supply heat to the thermopile, and since the earliest stages of the observed heat must depend largely on how they behave, the fact may be important. The 'inside' surface does not appear to differ very much from the middle in the size of its fibres.

In the double sartorius preparation ordinarily used the 'outside' is the surface which lies in contact with the thermopile. It seems unlikely that the layer of Ringer's fluid between the outer surface of this connective tissue and even the flattest and smoothest thermopile, is less than 1 or  $2\mu$  thick; so that an inert layer of 4 to  $10\mu$  must be present, hindering the heat flow. The value of  $b$  for thermopile P 6 (with a pair of muscles) is about  $10\mu$ , so that for recording the heat production of the contracting fibres its effective value is raised to 14 to  $20\mu$ . The flow of heat from muscle to thermopile involves the factor  $kt/b^2$ , and if  $b$  increases from 10 to 14 or  $20\mu$ ,  $b^2$  will increase from 100 to 200 or 400. Thus, the non-contracting layer will slow the response of the thermopile itself 2 to 4 times.

An obvious step would be to use the muscles the other way round, i.e. 'inside in'. On their inner surface there is nothing like a continuous layer of fascia, and apart from shreds of connective tissue left by dissection, and the Ringer's fluid which these might retain, the muscle fibres would be in direct contact with the thermopile face. The difficulty about this is to reverse a pair of muscles. The bone at the pelvic end would have to be sliced down the middle, and its two halves reversed and held in such a way that the muscles lay on the thermopile with the same precision and regularity as is achieved with the ordinary preparation. If they did not, irregular masses of fluid would be confined between them and the thermopile face, the muscles would flop about when stimulated and the results would be unreliable. An attempt was made to work this way with a double sartorius reversed, but abandoned.

It is easy to work with a single sartorius 'inside in', and experiments have shown that the deflexion obtained on stimulation is rather more rapid than with the same single muscle the other way round. No advantage, however, is gained. With a single muscle the whole of the thermopile has to be warmed from one side, not one-half of it, so that the effective value of  $b$  is not 10 but  $20\mu$ . Unavoidable inert material and fluid added to this would make the deflexion slower than with the double muscle 'outside in'.



## 5. TIMING AND DISPLAY

*Timing*

The timing of events was effected by means of a revolving contact breaker with three arms operating three keys. The contact breaker revolved once in 10 sec., and the metal drum on which the arms were set was engraved in 1000 divisions, each corresponding to 10 msec. By careful setting, short intervals could be adjusted to within 1 msec.; their duration could be measured on the cathode-ray tube, and once set they were consistent to less than 1 msec.

The opening of the first key  $K_1$  broke the current through two rapid relays arranged with their coils in series. The opening of one relay brightened the beam of the cathode-ray tube and started the sweep. The opening of the other relay provided a condenser discharge to stimulate the muscle. These events occurred within a fraction of 1 msec. of one another.

The second key  $K_2$  was not used when single shocks were employed.

The opening of the third key  $K_3$  operated a relay which caused a contact to be made closing the first relay referred to under  $K_1$ , so darkening the beam and stopping the sweep. In the interval between  $K_1$  and  $K_3$  the beam was bright and sweeping.

When currents of known duration were required, whether for a tetanus or for heating, these were provided by  $K_1$  and  $K_2$ . The opening of the second relay of  $K_1$  broke the contact of a Carpenter relay (type 3E1); the opening of  $K_2$  made that contact again. The contact short-circuited the stimulating or heating current. During the interval between  $K_1$  and  $K_2$  the current passed through the muscle. The Carpenter relay is a remarkable instrument, allowing a contact to be *made* very quickly and accurately and without a trace of 'chatter'. The interval  $K_1$ - $K_2$  once set was consistent within less than 1 msec.

*Display*

Various arrangements were tried, the one finally adopted being due to Attree (1949). At the moment when the stimulus was applied the beam brightened and the sweep started, continuing until stopped by  $K_3$  (see figure 6*b*). A nearly linear repetitive time-base, at any desired speed and with negligible fly-back time, allowed the record of the deflexion to be spread out as required. A fourth key  $K_4$  permitted the speed of sweep to be reduced to one-quarter, if desired, at any moment during a deflexion. Time marks were provided on the record by modulating the beam, either in gaps or spots, at any frequency required; 50 time-marks per second were usually sufficient.

Photographic records were made with a fixed quarter-plate camera with dark slides. The room was fairly dark and, with the beam off, the plate or paper could be exposed as long as necessary while the revolving contact breaker moved round to open the keys. Before  $K_1$  was opened a key operated by hand momentarily brightened the beam without starting the sweep, in order to give zeroes of time and deflexion (see figure 6*a, b*).

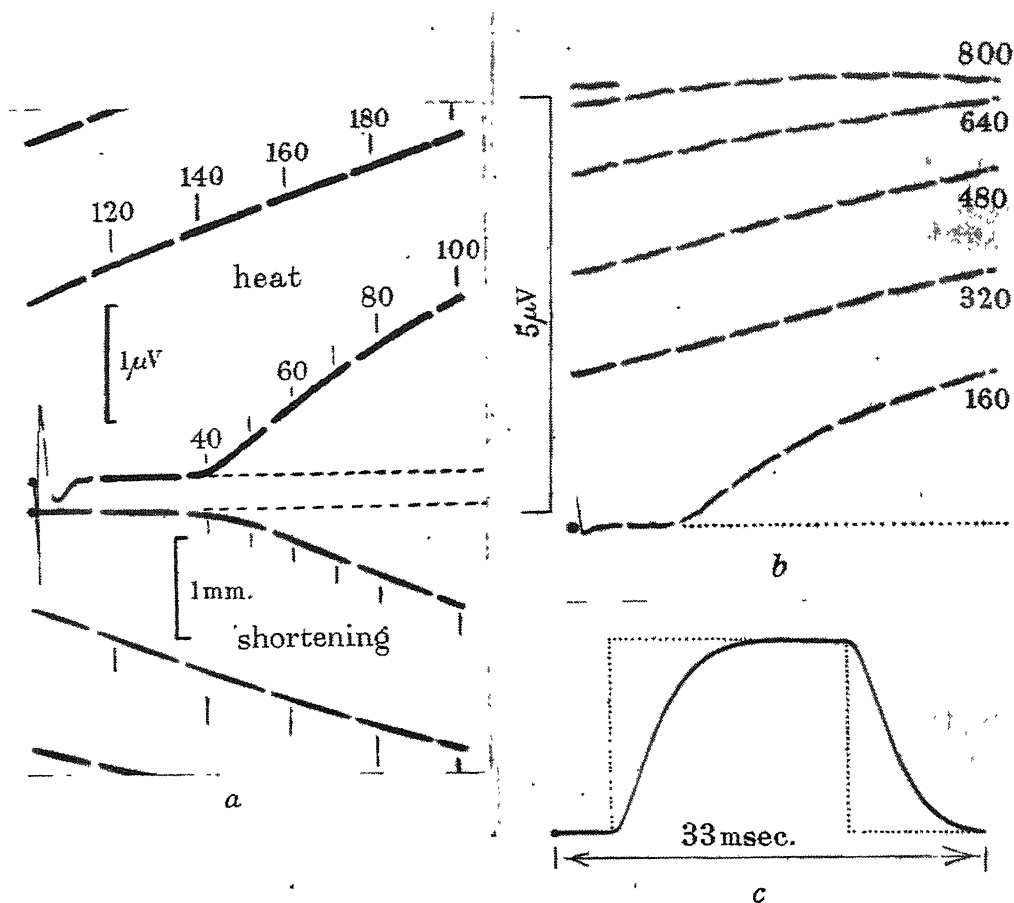


FIGURE 6. *a*. Simultaneous records of heat and shortening in the early stages of an isotonic twitch at  $0^{\circ}\text{C}$  of a pair of semi-membranosus muscles of a toad. Thermopile P7,  $1430\mu\text{V}/1^{\circ}\text{C}$ . Muscles 143 mg., load 4.3 g. Numbers, time in msec.; time gaps every 20 msec. *b*. Record (five sweeps) of the heat production in an isotonic twitch at  $0^{\circ}\text{C}$  of a pair of sartorius muscles of a toad. Total heat  $3.2 \times 10^{-3}\text{ cal./g.}$  Numbers, time in msec.; time gaps every 20 msec. In *a* and *b* note the initial point (zero of time and deflexion) at the left-hand bottom corner, and the leak of the stimulus. *c*. Deflexion of galvanometer, as used in the records of *a* and *b*, in response to a constant current pulse (dotted line) lasting 18 msec. The galvanometer introduced a delay of about 3.5 msec. in the heat records.

## 6. THE STIMULUS

In recording the earliest stages of the heat production it is necessary that the galvanometer should not be badly deflected by a leak of the stimulus. Even if the insulation is perfect the thinness of the mica sheet (10 to  $15\mu$ ) still gives a substantial electrical capacity between muscle and thermopile and so causes a momentary difference of potential to be set up between regions of the thermopile opposite portions of the muscle between which a current is flowing. This produces a momentary flick of the galvanometer which, if no precautions are taken, may be large enough to obscure the early deflexion due to heat. The trouble can be largely

diminished (i) by using three electrodes, a cathode at the middle and anodes at the ends, so causing the ends of the thermopile to remain more nearly at the same potential when a shock is applied to the muscle, (ii) by earthing the two anodes, and (iii) by keeping the insulation as near perfect as possible. Even so, disturbances occur when a one-way shock is applied.

If the shock is rapidly reversed, so that the same quantity of electricity flows in the muscle in both directions, the disturbance produced by current one way is rapidly reversed by current the other way and the net disturbance after 2 or 3 msec. is greatly reduced. The circuit employed is shown in figure 7*a*.

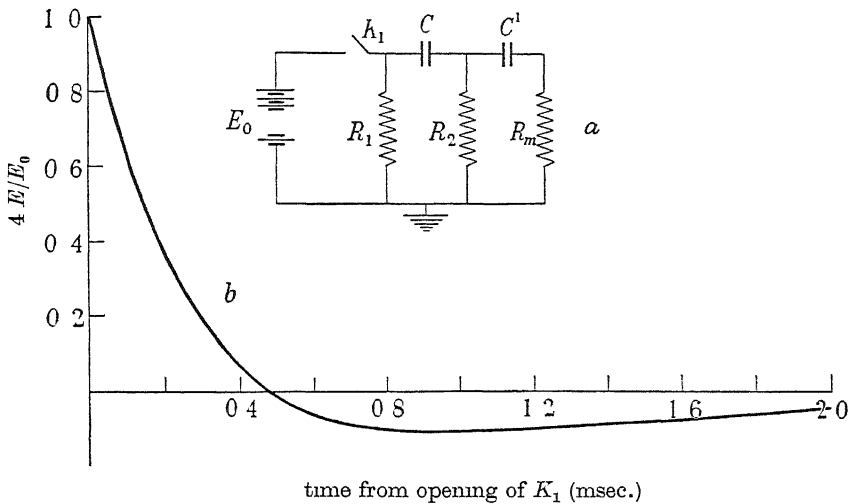


FIGURE 7. *a*. Circuit for single shock to muscle  $R_m$  on opening key  $K_1$ . *b*. Form of the two-way discharge on opening  $K_1$ , equation and constants in text.

The shock (a condenser discharge) is produced by opening the key  $K_1$ . In series with the muscle, represented by the resistance  $R_m$ , is a condenser  $C'$ ;  $C'$  ensures that the total current which passes through the muscle is nil. The time course of the potential  $E$  applied to the muscle is then

$$E = \frac{E_0}{1 + R_1/R_m + R_1/R_2} \left( \frac{b}{b-a} e^{-t/a} - \frac{a}{b-a} e^{-t/b} \right),$$

where  $a$  and  $b$  are

$$\frac{1}{2}[(R_2 + R_m) C' + (R_2 + R_1) C \mp \sqrt{\{(R_2 + R_m) C' - (R_2 + R_1) C\}^2 + 4CC'R_2^2}].$$

The form of  $E$  is shown in figure 7*b*. It crosses the base-line at time

$$t = \frac{\log b/a}{0.434(1/a - 1/b)}$$

and reaches its negative maximum at twice this value. The area is the same above as below the line; the first phase stimulates, the second phase reverses any disturbance of the record or polarization of the muscle and electrodes.

The resistances and capacities must be chosen to give an appropriate shape to the discharge, they depend upon  $R_m$ , the resistance of the muscle. For a pair of

sartorius muscles of the usual size the resistance between the end electrodes is 10,000 to 20,000  $\Omega$ : using a central cathode and anodes at the ends, it is 2500 to 5000  $\Omega$ . Figure 7 *b* is drawn for  $R_m = 5000 \Omega$ ,  $R_1 = 5000 \Omega$ ,  $R_2 = 2500 \Omega$ ,  $C = 0.1 \mu\text{F}$ ,  $C' = 0.05 \mu\text{F}$ , and  $E$  then has the form

$$E = \frac{1}{4}E_0(1.59 e^{-t/0.305} - 0.59 e^{-t/0.82}),$$

where  $t$  is in msec. The current has effectively ceased by 5 msec. The deflexion recording heat production is never apparent at  $0^\circ \text{C}$  before about 15 msec. after the shock, so the passage of the stimulating current and its reversal should leave the galvanometer quiet some time before the heat deflexion begins. Figure 6 *a*, *b*, shows the leak of the stimulus at the beginning of the record.

There might be some advantage in stimulating the muscles through their nerves (Hartree 1929), though this would require two more pairs of electrodes, and the total delay in propagation and at the neuro-muscular junction would be greater than in direct stimulation. The chief objection, however, would be that when considerable shortening occurred the nerves might be displaced or damaged. No attempt has been made to work in this way.

## 7. THE ANALYSIS OF RECORDS

The thermopile and galvanometer now in use are so quick that the records themselves exhibit the chief phenomena without analysis, e.g. the rapid onset of heat production after a shock, the effect of allowing a muscle to shorten and the heat derived from mechanical energy when a muscle relaxes under tension or load. For the finer resolution, however, of the several processes and for greater precision in determining their course and magnitude, an analysis of the records is still necessary. This is due to three facts:

(1) The galvanometer, although very rapid, is not instantaneous, and the lag it introduces into the records is not negligible.

(2) The thermopile, although very thin, is not warmed up instantly by the layer of muscle in immediate contact with it; its heat capacity, though small, requires heat to be conducted from a distance and conduction through muscle is slow.

(3) Heat loss, although small in the time involved (1 or 2 sec. for contraction and relaxation), is not negligible except in the early stages and must be allowed for.

In the past, records were analyzed by a numerical method based on the use of an experimental 'heating control'. The muscle was heated artificially during a known short interval by a strong high-frequency current (say 100 kc./sec.) which does not stimulate it; or, for security against casual stimulation, the muscle could first be made inexcitable by soaking in Ringer's solution containing 10 times the usual potassium ion concentration. A record was made exactly as when the muscle produced heat in response to a stimulus. The one record was then used to analyze the other.

This method worked well with the older thicker thermopiles. With them the extra delay due to the layer of connective tissue and fluid between muscle fibres and thermojunctions (see § 4) was relatively unimportant. With the present

instruments, however, a heating control of the old kind would rise much too rapidly, since the inert material and fluid would be warmed as well as the muscle by the heating current. To take full advantage of the quicker instruments it was necessary to allow for the unavoidable addition to the heat capacity of the thermopile, provided by the inert material and fluid. In the attempt to do so it was found that the simplest and most accurate procedure was to break down the analysis into its several components in the following order.

7.1. *Lag due to galvanometer.* In a recent paper (Hill 1948 c) it was shown that the effect of galvanometer lag, in recording a gradually changing current, is—except at very short times—simply to delay the whole curve of deflexion by a constant small amount. Given the deflexion of the galvanometer to a constant current suddenly introduced in its circuit (figure 6c), that delay could be easily calculated. With the galvanometers used in the present work it was  $1\frac{1}{2}$  to 4 msec. The necessary allowance is made by changing the zero of time by the calculated small amount.

At very short times with a rapidly changing deflexion rather greater accuracy is obtained by analyzing the deflexion by a 'control' consisting of the galvanometer response to a constant current suddenly introduced. Before long the time lag is found to become constant.

7.2. *Heat loss* occurs along the wires of the thermopile and (to a lesser degree) to the surrounding air. Its rate is proportional to the difference of temperature between the muscle and its surroundings, i.e. to the deflexion at the moment. For a larger muscle the rate of temperature loss is less; depending on the size of the muscle its value was from 3 to 8 % per sec. The coefficient of temperature loss was obtained by heating the muscle artificially for a short time with a high-frequency current and observing the subsequent decrease of the deflexion. Allowance for heat loss is made numerically; it is of little importance during the first 0.2 sec.

7.3. *Lag due to thermopile.* By numerical integration of equation (1) in § 3 it is possible to calculate the course of the current produced in a thermopile by heating a muscle on it for a known time, say 5 msec. Taking the equivalent half-thickness of the thermopile as  $10\ \mu$  and adding to it a quantity representing the thickness of the connective tissue and fluid between muscle fibres and thermopile, we obtain the value of  $b$  for the calculation. The exact thickness of the inert material cannot be known, but no great error is involved in taking it as  $5.8\ \mu$ ; this precise value was chosen because it made  $b^2/4kt$  equal to  $\frac{1}{2}t$ , if  $t$  was in msec., which was convenient for calculation.

The calculated 'heating control' (thermopile current) for 5 msec. heating with heat 1000 is as follows:

time (msec.)	0	5	10	15	20	25	30	35	40	45	50
deflexion	0	462	711	776	811	833	848	860	870	878	885
time (msec.)	55	60	65	70	75	80	85	90	95	100	
deflexion	890	895	900	904	907	910	912	914	916	918	

In a calculation up to 0.1 sec. it is unnecessary to take account of equation (2), § 3, which does not affect the result until much later.

With a calculated heating control of this kind the analysis is carried out in the usual way. The interval, 5 msec., is about the shortest that can usefully be employed; a 10 msec. heating control is practically as good, and much less trouble, for the early analysis. For the later analysis 20, 40 or 80 msec. intervals are sufficient.

For longer analyses, the finite thickness of the muscle begins to affect the result between 0.5 and 1.0 sec., and the small modification embodied in equation (2) must then be adopted.

Various 'heating controls' were prepared in this way, for use as appropriate.

A long analysis carried out in the usual way by successive subtractions is laborious, and a much simpler procedure has been found (Hill 1949*d*). Let  $z_1, z_2, z_3, \dots$  be the observed values of the deflexion required to be analyzed at equal intervals of time from the start. Then the total heat  $H_r$  up to the end of the  $r$ th interval is given by

$$H_r = a_1 z_r + a_2 z_{r-1} + a_3 z_{r-2} + \dots + a_r z_1. \quad (3)$$

Here  $a_1, a_2, a_3, \dots$  are constants which can be calculated once for all for a given interval of analysis. Calculations from equation (3) are quickly made with an adding machine. For 40 msec. intervals ( $b = 15.8 \mu$ ) the values of  $a_1, a_2, a_3, \dots$  (all multiplied by 1000) are as follows:

1297, -207, -12, -10, -8, -6, -4, -3, -3, -2, -2, -2, -2, -1, -1, ....

The method of calculating the  $a$ 's is described in the paper referred to.

For analyzing at the start in very short intervals the method of successive subtractions is to be preferred, since it allows discretion in accepting small remainders, to cover small irregularities in the numbers analyzed. Equation (3) is automatic and allows no remainders, and with very short intervals is apt to produce an oscillatory result. For longer intervals, however, it works perfectly, and has the great advantage that any value of  $H_r$  can be calculated without calculating all the others.

In working with a single muscle (which has to warm the whole thickness of the thermopile) the value taken for  $b$  must be greater, namely, 20  $\mu$  plus the allowance for inert material between muscle fibres and thermopile. This makes the calculated 'heating control' considerably slower and the early analysis less sharp.

## 8. SIMULTANEOUS RECORDING OF THE MECHANICAL RESPONSE

The exact correlation of the heat with the mechanical changes, tension or shortening, throughout a twitch required that the latter should be recorded simultaneously in the same photograph without friction or delay. A double-beam cathode-ray tube was used, the second beam being operated by an amplifier connected to a simple 'strain-gauge' (for tension) or a twin rectifier photocell (for shortening). The strain-gauge has been described elsewhere (Hill 1949*e*); it consists of a single straight wire (nickel-chromium, 60  $\mu$  diameter) forming one arm of

a resistance bridge. For recording the shortening, a light dural lever mounted on miniature ball bearings moved between a lamp and the double photocell, casting a shadow on the latter. The two cells were arranged in opposition, movement of the shadow causing a change in the current produced by them, which operated the amplifier and the beam. The lever was so light that its inertia was negligible at the speeds involved, and friction in the ball bearings was very small. The deflexions for heat and mechanical response were arranged to be in opposite directions on the records and the starting-point and the time marks were the same for both. An example of simultaneous recording of heat and shortening, at high magnification, is given in figure 6*a*.

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# The onset of contraction

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The early heat production during the onset of a muscle twitch has been determined with the greatest precision possible for comparison with the mechanical response. When allowance is made for the time taken in propagation the heat is found to start off at its maximum rate. Its rate falls gradually to a constant value, while the muscle continues to shorten uniformly then decreases to zero as shortening draws to an end. The heat occurs in two separate processes, those of activation and shortening respectively. The heat of activation has well started before shortening is detected by ordinary methods. The heat of shortening runs parallel to the shortening.

There is no sign of negative heat production at any stage of contraction. If endothermic processes occur they are exactly masked by exothermic ones.

The latent period of the activation heat is about 10 msec. at  $0^{\circ}\text{C}$  in frog's muscle, about 25 msec. in toad's muscle. These, with an ordinary value of  $Q_{10}$ , would correspond at  $20^{\circ}\text{C}$  to about 2 and 5 msec. respectively.

Various physical methods are discussed of examining the rapid processes that occur during contraction. Chemical methods are inadequate in speed and sensitivity to give direct evidence of the nature and sequence of events occurring in a twitch. Theoretical conclusions from experiments on muscle extracts, without critical comparison with the behaviour of living muscle, may lead to confusing results.

## INTRODUCTION

In a recent paper (A. V. Hill 1949) it was shown that the initial heat production of skeletal muscle in a twitch can be resolved into two parts, heat of activation and heat of shortening, the former at a given temperature being little, if at all, affected by anything except the fact of stimulation, the latter being directly proportional to the change of length. The purpose of the investigation described in the following pages was:

(1) To determine with the greatest possible precision whether there is any sign of an endothermic reaction at any time during contraction and relaxation (as distinguished from recovery). This obviously required an equipment rapid enough to follow events occurring in a few milliseconds and sensitive enough to work with the elementary unit of muscular response, the single twitch. Much more heat indeed is produced in a tetanic contraction, but it was no good complicating the inquiry by superimposing a number of similar processes. A maximal tetanus of a frog's sartorius at  $0^{\circ}\text{C}$  is given by 25 shocks/sec. or less, and even the second of a series of shocks at this rate would fall when the heat production due to the first was already 20 to 30 % complete. Its incidence would entirely obscure the simple character of the relation to be observed.

(2) To push back the detection and measurement of the heat production as early as possible, (a) in order to find out whether the heat of activation and the heat of shortening could be clearly separated in their earliest stages, and (b) for



comparison with the most rapid and sensitive methods of detecting and measuring the mechanical response.

The answer to the first question can be given at once. Many experiments have been made under a variety of conditions; not once has there been any good evidence of a negative heat production during contraction or relaxation. If endothermic processes do occur they are masked by exothermic ones. It is easy to produce apparent signs of negative heat, e.g. by using an 'unprotected' thermopile and an inconstant thermostat, or by injuring the part of the muscle away from the thermopile so that heat produced near the thermopile is later conducted outwards to warm the inactive region. But by reversing a muscle on the thermopile such appearances due to non-uniformity can be disposed of. If negative heat production does occur at any stage during contraction its amount must be less than 1 % of the total initial heat. The earliest phase of activity is strongly exothermic.

All the experiments described have been made at 0° C. The single twitch of a frog's muscle at 0° C is actually at least as strong as at a higher temperature (Hartree & Hill 1921, p. 138), and occurs much more slowly, so that a large gain in the relative speed of one's instruments is obtained without any sacrifice of sensitivity. There is no objection to a low temperature; contraction, relaxation and recovery occur normally, and a muscle maintains its activity unimpaired for long periods (hours or days) if well treated. Any suggestion that a frog's muscle (or many other muscles) does not work 'normally' at 0° C is contrary to experience. When the resolution of events following one another with great rapidity is required it would be foolish not to accept the very great advantage of working at a low temperature. Moreover, from the point of view of avoiding extraneous disturbances, a thermostat consisting of well-stirred ice and water is the best possible one. The mechanical efficiency of frog's muscle is as great at 0° C as at higher temperatures and approximates to that of human muscle at 37° C, while the maximum force exerted in a twitch may be 1 to 2 kg./cm.<sup>2</sup>. It is true that a frog cannot jump at a low temperature, but that is simply because its muscles shorten too slowly to lift it off the ground. An English toad (*Bufo bufo*) can scarcely jump at ordinary temperatures, and for the same reason; but its muscles are very strong.

Varga (1946), in Szent-Györgyi's laboratory, recently examined the relation between temperature and amount of shortening in slices of muscle treated as follows. The muscle was frozen in dry ice and cut into narrow thin strips. These were then placed in distilled water at various temperatures and shortened various amounts. From the amount of shortening, assuming a reversible chemical equilibrium to exist (for which no evidence was given), an 'equilibrium constant' was calculated:

$$K = \frac{\text{shortening observed}}{\text{maximal shortening} - \text{shortening observed}}.$$

From the variation of  $K$  with temperature the 'heat of reaction' was calculated as -53,500 cal., and from  $K$  the 'free energy' was deduced at various temperatures. At 0° C the 'free energy' so calculated for a frog's sartorius was very small, about +700 cal., compared with 5000 cal. at 20° C; from which it was deduced by Szent-Györgyi (1947, p. 49) that a frog's muscle can contract only weakly at 0° C,

which is quite untrue. The quantities found were attributed to the reaction by which actomyosin goes over, under the influence of ATP, from the relaxed to the contracted state, the breakdown of ATP providing free energy later for relaxation.

The whole procedure involves a series of unjustified assumptions; to apply its results to the case of normal muscle would be highly precarious. It provides, however, the only evidence (i) that an endothermic reaction does occur in muscular contraction, or (ii) that the 'free energy' of shortening at 0° C is very small.

#### THE EARLY HEAT PRODUCTION IN A TWITCH

The mechanical response of a frog's sartorius in a twitch at 0° C is not revealed by ordinary methods, even those involving a minimum of friction or inertia, before about 20 to 25 msec. after the stimulus. With a piezo-electric method of very high sensitivity and extremely small inertia, similar to that employed by Sandow (1944, 1947) in his work on 'latency relaxation', recording the local response at the stimulated point, Abbott & Ritchie (1948) found in frog's muscle at 0° C that the initial drop of tension was just detectable about 7 msec. after a shock. The minimum occurred at about 15 msec.; at 18 msec. the tension had reached its initial value and was rising rapidly.

Myothermic equipment cannot be made sensitive enough to detect and measure the heat in the very limited region directly excited by a shock. For sufficient sensitivity a good many thermocouples must be used, making contact with about 1 cm. of muscle. If the shock is applied to the middle of this the contraction, being propagated at about 35 cm./sec., requires about 14 msec. to cover the whole thermopile. For technical reasons it would be very difficult to apply multiple stimulation at a number of electrodes all along the thermopile, and one has to be content with stimulation at one point. This means that the heat production observed starts off at a very low rate, occurring at first only in the immediate region of the electrode and gradually increasing in rate as the contraction is propagated. An approximate allowance can be made, as we shall see, for the time taken in propagation, but this still leaves an element of uncertainty as to the precise moment at which the heat starts. With the piezo-electric method of recording the mechanical response precisely at the stimulated point no such uncertainty occurs. When simultaneous records are made of heat and shortening, the latter may be even more affected by propagation time, since the muscle is considerably longer than the thermopile; in a 30 mm. muscle stimulated at a single point 8 mm. from one end, the other end would begin to shorten about 60 msec. later than the region under the electrode. This, in fact, is an overestimate, because a good many of the muscle fibres are bound to be stimulated in the regions of the anodes, and stimulation of nerve branches is certain to occur; the effect of propagation on the form and onset of contraction must be considerable, but under the conditions of the present experiments cannot be accurately allowed for.

In figure 1, curve *a* is the mean of twelve records, in close agreement, of the heat deflexion in a single isotonic twitch at 0° C of a pair of frog's sartorii under 2.6 g. load, subjected to a three-times maximal shock at time zero. The total initial heat

was about  $2.5 \times 10^{-3}$  cal./g., the individual maxima varying from the mean by  $\pm 2\%$ . Curve *b* represents the result of analyzing curve *a*, at first in intervals of 5 msec., later of 10 msec. Curve *a'* is the average heat deflexion in eleven similar experiments on eleven pairs of frog's muscles. The mean curve must be rather accurate, since each experiment included about twelve deflexions, read every 10 msec. up to 200 msec., a total of about 2600 points. Curve *b'* represents the analysis of curve *a'*.

In figure 2 the rate of heat production is plotted, instead of the total heat, *d* corresponding to deflexion *a*, figure 1, *d'* to deflexion *a'*. The blocks shown are the amounts of heat in successive 5 msec., or 10 msec., intervals as obtained by analysis. The results of all the experiments made conform to this type. The rate of heat production seems to start about 10 msec. after the shock and to reach a maximum about 20 msec. later; it then falls off gradually, first becoming nearly constant, then dropping gradually to zero at about 400 msec. as the shortening ends. The time from the start to the peak in *d* is 21 msec., in *d'* 18 msec. The distance of the stimulating cathodes from the last elements of the thermopile was 6.6 mm. in either direction. If we assume that the duration of the initial rise is the time occupied in the propagation of contraction over the thermopile, the velocity of propagation for the average of the eleven experiments is  $0.66/0.018 = 37$  cm./sec. According to Eccles, Katz & Kuffler (1941, table 3) the conduction velocity in the fibres of an Australian frog's sartorius at  $20^\circ\text{C}$  is about 1.6 m./sec.; with their observed  $Q_{10}$  this would be about 36 cm./sec. at  $0^\circ\text{C}$ .

It is reasonable, therefore, to assume that the rise in heat rate at the start is due to the propagated spread of the contraction from the stimulating electrode over the thermopile. To test this assumption directly the following experiment was made. A single shock was applied to a pair of muscles on the thermopile either (*a*) as usual, the cathodes being over the region of the thermopile in which the heat was measured, or (*b*) between a cathode *X* at the tibial tip of the muscle and the nearer anode *A'*. Sixteen records were made for (*a*) and eight for (*b*), interspersed with one another; agreement in each group was good and the mean deflexions were drawn and analyzed.

In case (*b*), if excitation was at *X* the distance of propagation to the nearest point of the thermopile was 18 mm.; if excitation was at *A'* it was 6 mm.; possibly it occurred also at various points in between (the shock being three times maximal), and some nerve fibres may have been excited, with varying delay, before the contraction so set up reached the thermopile.

The outcome was clear. The deflexions were obviously different in their earlier stages, (*b*) being considerably delayed and rising less rapidly after its start. Later, however, the deflexions became identical in shape, (*b*) merely being about 30 msec. later than (*a*). Analysis gave the following results (*H* being the total initial heat):

	( <i>a</i> )	( <i>b</i> )
time, shock to first appearance of heat	10 msec.	28 msec.
peak of heat-rate	sharp, at 30 msec.	blunt at about 57 msec.
fall after peak	rapid	slower
maximum heat-rate	11.6 <i>H</i> /sec.	7.8 <i>H</i> /sec.
plateau around 100 msec.	4.0 <i>H</i> /sec.	4.0 <i>H</i> /sec.

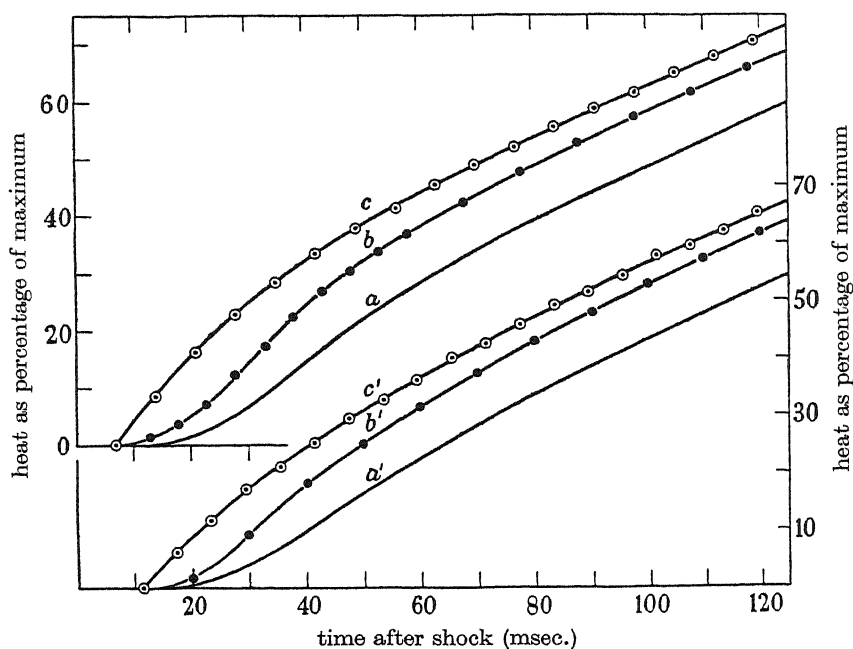


FIGURE 1. The earlier stages of the initial heat production of frog's muscle at  $0^{\circ}\text{C}$ , in an isotonic twitch under small load. *a*, record (mean of twelve twitches of one muscle); *b*, analysis in 5 and 10 msec. intervals; *c*, heat after allowance for time taken in propagation; *a'*, mean record for eleven muscles; *b'*, analysis in 10 msec. intervals; *c'*, as *c* above. *c* and *c'* represent what *b* and *b'* would have been, had the muscles been stimulated simultaneously all over.

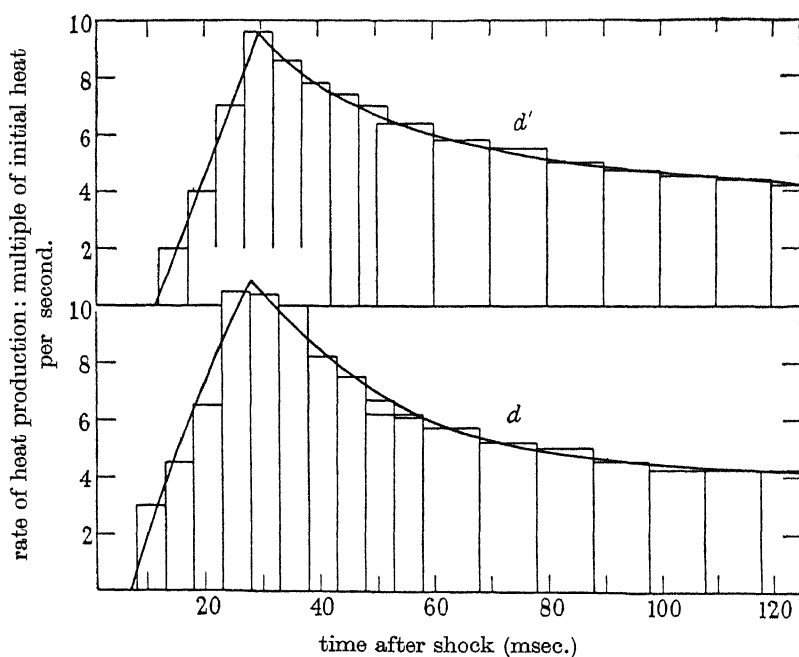


FIGURE 2. Rate of heat production, results of analysis, *d* of record *a* and *d'* of record *a'* in figure 1; used for calculation of allowance for time taken in propagation as described in the text.

For case (a), the curve of heat-rate was similar to the curves of figure 2, while (b) differed from (a) exactly as predicted. First, there was delay in arrival of the wave of contraction at the thermopile and therefore in the first appearance of heat; secondly, since the wave was moving in one direction only over the thermopile, instead of spreading out in two directions from the middle, the interval between first appearance and peak of heat production was greater; thirdly, since the heat-rate rapidly diminishes at first at an active point (see figure 2) the observed heat-rate over the whole thermopile had a lower maximum when propagation took longer, and fell off more slowly after; and fourthly, after propagation had been completed the time course of the heat production became identical in the two cases.

Katz & Kuffler (1941, figure 1) give a diagram of the motor innervation of the frog's sartorius. From it we can deduce that a stimulus applied about  $8\frac{1}{2}$  mm. (one-quarter of the length) from the pelvic end may indeed excite nerve fibres near their end-plates in that region, but that the stimulation of these or any other nerve fibres would scarcely quicken the arrival of the contraction at the region where the heat was measured in the present experiments, viz. between 2 and 15 mm. from the pelvic end. According to Eccles *et al.* (1941, table 3) the 'neuromuscular latency' in the frog's sartorius at  $20^{\circ}\text{C}$  is about 1.3 msec., which, with their observed  $Q_{10}$  of 2.1, would be about 6 msec. at  $0^{\circ}\text{C}$ . A delay of 6 msec. at the junction would be a serious handicap to nerve transmission in competing with conduction along the muscle fibre (even at only one-tenth of the velocity) for first arrival at a point a few mm. away from the stimulating cathode. Let us assume that activity was set up gradually, over the  $\pm 6.6$  mm. of the part of the muscle in which the heat was measured, by propagation along the muscle fibres alone.

Suppose that a wave of contraction is started at the stimulating cathode and propagated with velocity  $v$ . Let the total distance of propagation be  $l$  either way and the time of propagation  $\tau$ : then  $v = l/\tau$ . Let  $y = F(\theta)$  be the total heat production at any point, reckoned per cm. length of muscle, up to time  $\theta$  after it was stimulated. This would be the form of the heat production if the whole muscle could be excited simultaneously. Then at a point distant  $x$  cm. from the electrode the total heat production per cm. length of muscle up to time  $t$  after a shock must be zero until  $t = x/v$ , and after that  $F(t - x/v)$ . The total heat production up to time  $t$  in length  $2l$  of muscle will then be

$$H(t) = 2 \int_{x=0}^{x=l} F(t - x/v) dx.$$

Differentiating with respect to  $t$ ,

$$\begin{aligned} H'(t) &= 2 \int_{x=0}^{x=l} F'(t - x/v) dx = -2v \left[ F(t - x/v) \right]_{x=0}^{x=l} \\ &= 2v [F(t) - F(t - l/v)]. \end{aligned}$$

Writing  $l/v = \tau$  and  $v = l/\tau$ , this becomes

$$2lF(t) = \tau H'(t) + 2lF(t - \tau). \quad (1)$$

Now  $H'(t)$  is the observed rate of heat production, at time  $t$ , of the whole muscle on the thermopile. If, therefore, we know  $\tau$  we can calculate  $2LF(t)$ , the whole heat that would have been given out up to time  $t$ , had excitation been simultaneous over the whole muscle, by working out successive values of  $2LF(t)$  from quantities which have been observed. The process can be simplified as follows:

$$\begin{aligned} 2LF(t) &= \tau H'(t) + 2LF(t-\tau), \\ 2LF(t-\tau) &= \tau H'(t-\tau) + 2LF(t-2\tau), \\ 2LF(t-2\tau) &= \tau H'(t-2\tau) + 2LF(t-3\tau), \end{aligned}$$

etc., continuing till the last term on the right is zero. Adding both sides and cancelling common terms we get

$$2LF(t) = \tau[H'(t) + H'(t-\tau) + H'(t-2\tau) + \dots]. \quad (2)$$

From curve  $d$ , figure 2, the rate of heat production was read off every 7 msec., and the value of  $2LF(t)$  calculated from equation (2) taking  $\tau = 21$  msec. The result is as follows,  $H'$  being as a multiple of the maximum initial heat per sec.,  $2LF(t)$  being as a fraction of the maximum initial heat:

$t$	7	14	21	28	35	42	49	56	63
$H'(t)$	0	4.1	7.7	10.9	9.4	8.2	7.1	6.2	5.7
$2LF(t)$	0	0.086	0.162	0.229	0.284	0.334	0.378	0.413	0.454
$t$	70	77	84	91	98	105	112	119	126
$H'(t)$	5.3	5.0	4.8	4.6	4.5	4.4	4.3	4.2	4.2
$2LF(t)$	0.489	0.519	0.554	0.585	0.613	0.647	0.675	0.701	0.735

The result is given in curve  $c$ , figure 1.

A similar calculation was made from curve  $d'$ , figure 2. The values of  $H'(t)$  were read off every 6 msec. and those of  $2LF(t)$  calculated from them, taking  $\tau = 18$  msec.:

$t$	11½	17½	23½	29½	35½	41½	47½	53½	59½	65½
$H'(t)$	0	3.4	6.5	9.6	8.4	7.6	6.9	6.4	6.1	5.8
$2LF(t)$	0	0.061	0.117	0.173	0.212	0.254	0.297	0.328	0.364	0.401
$t$	71½	77½	83½	89½	95½	101½	107½	113½	119½	125½
$H'(t)$	5.5	5.3	5.1	4.9	4.7	4.6	4.5	4.4	4.3	4.2
$2LF(t)$	0.426	0.459	0.493	0.515	0.544	0.576	0.595	0.623	0.653	0.672

The result is given in curve  $c'$ , figure 1.

The fact that the muscle began to shorten at about the moment when the heat production commenced has been neglected in this calculation. In 20 msec. of propagation, the amount of shortening must have been a small part of 1 mm., and the only uncertainty is really that of the velocity of propagation in a muscle which is also shortening. The maximum velocity of shortening (under zero load) of a frog's sartorius at 0° C is about 1.3 times its length per sec. (Hill 1938), and the end of the recording region of the thermopile was about 1.55 cm. from the fixed pelvic end of the muscle. The maximum velocity of shortening here would be about  $1.3 \times 1.55 = 2$  cm./sec., and even that would not be reached immediately at the start. This is a small fraction of the velocity of propagation (30 to 40 cm./sec.), so its neglect is justified.

Curves *c* and *c'*, figure 1, represent what the heat deflexion would look like if the whole of the muscle could be excited together at time zero and if there were no lag in the instruments. Its maximum rate is at the start, about 10 msec. after the shock; from then onwards its rate decreases gradually, becoming nearly constant for a time, as we shall see, while the muscle is shortening uniformly, then dropping to zero as shortening ends.

Six similar experiments were made on toad's muscles at 0° C. The processes of contraction are slower in toad's muscles than in frog's, but otherwise the results were exactly similar; the heat started on the average 26 msec. after the shock, the

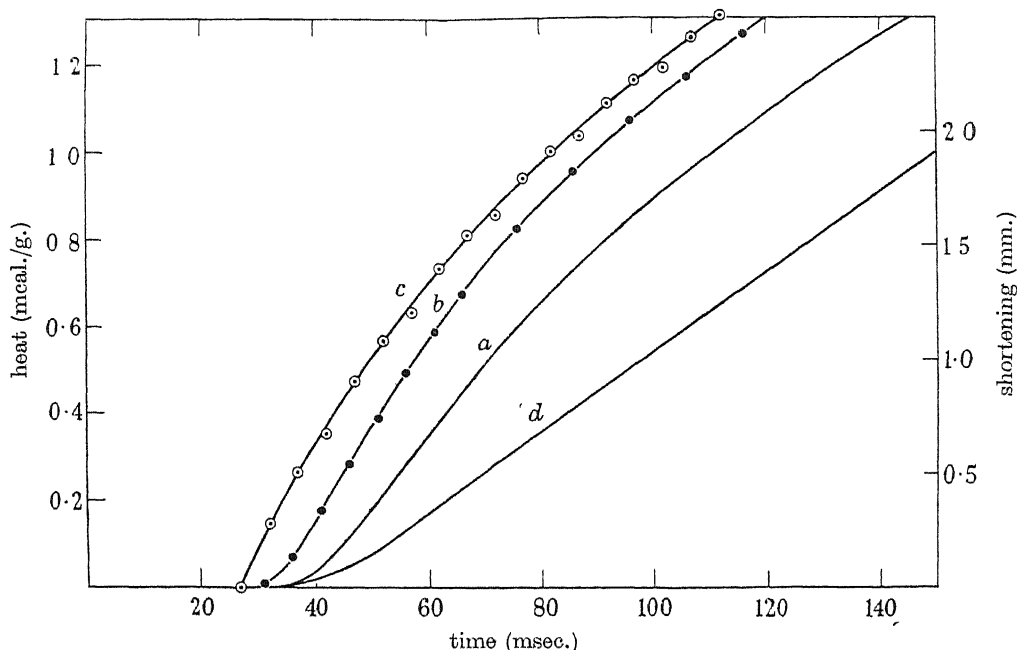


FIGURE 3. Simultaneous records, *a* and *d*, of heat and shortening respectively in the early stages of an isotonic twitch, of a pair of toad's semi-membranosus muscles (143 mg., 25 mm.) at 0° C under 4.3 g. load. Curve *b*, results of analysis of heat in 5 and 10 msec. intervals. Curve *c*, heat after allowance for time taken in propagation. *a* and *d* are the mean of six records. The total heat produced in the twitch was about 5 mcal./g. and the total shortening was about 11 mm.

individual delays being 24, 24, 25, 26, 27 and 30 msec. In these experiments the mechanical response was recorded at the same time as the heat. Figure 3 shows the heat deflexion (curve *a*) during the first 150 msec. of an isotonic twitch, and the shortening. The heat production after analysis is given in curve *b*. Figure 4 gives the direct results of the analysis, together with broken lines indicating the rate of heat production. Curve *c*, figure 3, shows the heat production after allowance for time taken in propagation, calculated from figure 4 in the manner described above. Figure 5, from another experiment, gives the heat (after analysis) and the shortening throughout an isotonic twitch in which the muscle shortened by 42% of its length.

The results given in figures 3 and 5 are typical of all such experiments made on toad's muscle. Figure 6, showing an experiment on frog's muscle, is exactly like figure 5 but on half the scale of time. For the reasons given above the curves of shortening cannot be corrected with any certainty for time taken in propagation, and it would be unfair to compare the curve of observed shortening with that of

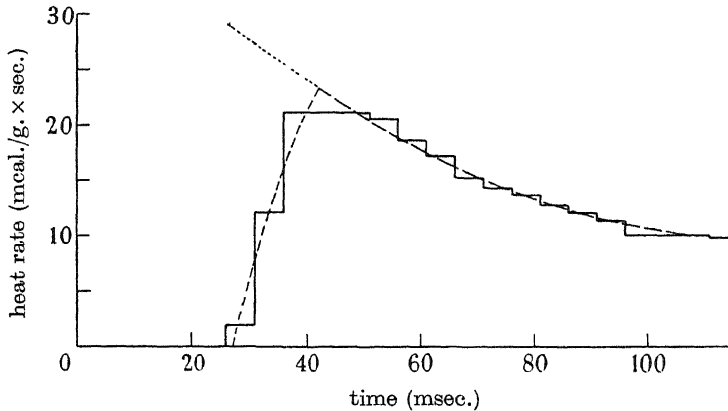


FIGURE 4. Rate of production. Results of analysis of record *a* in figure 3; used for calculation of allowance for time taken in propagation as described in text.

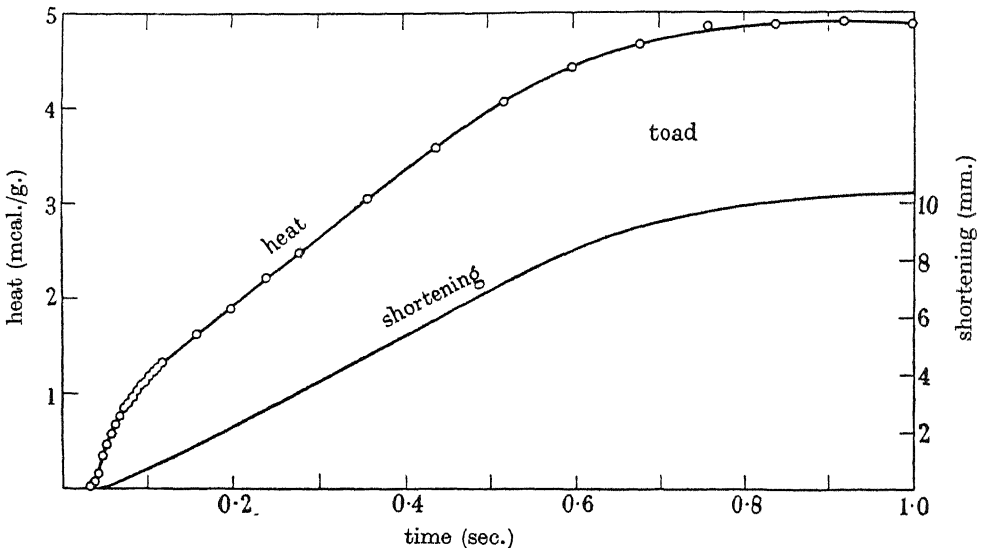


FIGURE 5. Heat and shortening simultaneously recorded in the isotonic twitch of a pair of toad's semi-membranosus muscles (132 mg., 25 mm.) at  $0^{\circ}\text{C}$  under 6 g. load. Circles, results of analysis in 5, 40 and 80 msec. intervals.

heat after allowance for propagation. The only thing to be done is to take both as observed, the actual shortening and the actual heat (after analysis). It is clear then, particularly from figures 5 and 6, that the heat starts earlier than the shortening and rises much more rapidly. Later on both curves become linear for a time, then both bend off to their maxima as shortening ends.



The heat consists of two parts, the heat of activation and the heat of shortening. The former provides the very early rapid deflexion, the latter comes in gradually as shortening gets under way. Accepting the interpretation that the early rise to a peak in figures 2 and 4 is due to propagation we conclude that the heat of activation starts off at its maximum rate suddenly, about 10 msec. after a shock in frog's muscle, about 25 msec. after in toad's muscle. Its rate then falls gradually to zero. The shortening heat begins later and runs parallel to the shortening, ending as shortening ends.

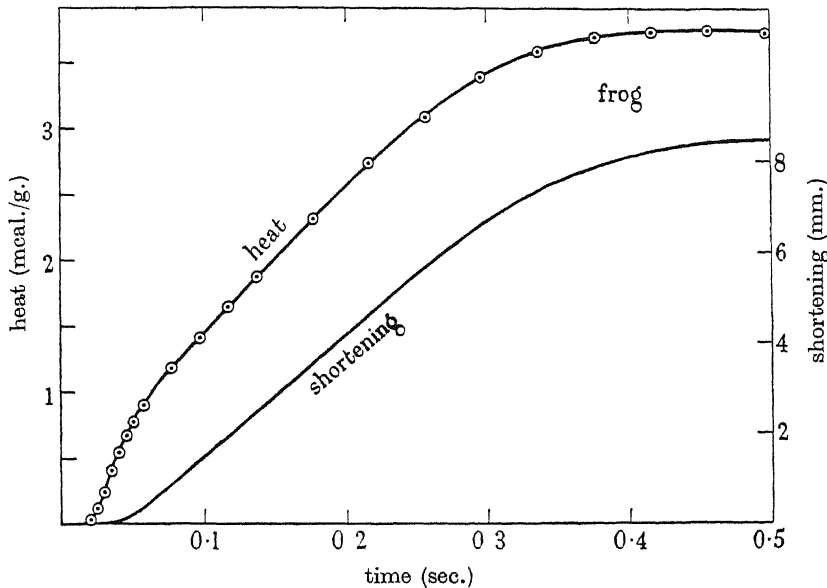


FIGURE 6. Heat and shortening simultaneously recorded in the isotonic twitch of a pair of frog's sartorii (120 mg., 31 mm.) at  $0^{\circ}\text{C}$  under 4.3 g. load. Circles, results of analysis in 5, 10 and 20 msec. intervals.

Concerning the nature of the delay at the start, one can only speculate. According to Eccles *et al.* (1941, p. 368), the interval between the first sign and the maximum of the muscle action potential excited from an end-plate has a value which at  $0^{\circ}\text{C}$  (with the usual temperature coefficient) would be about 7 msec. According to Katz (1942, p. 178) the interval for the change of electrical impedance would be about 5 msec. It does not follow that an interval of this order exists when a super-maximal shock is directly applied, though it presumably would as soon as the contraction begins to be propagated (i.e. during most of its passage along the thermopile). The shocks used in the present work were of short duration; the effective part of them was complete in less than 1 msec. At  $0^{\circ}\text{C}$  the activation process (as shown by its heat) starts about 10 msec. later than the shock in frog muscle, about 25 msec. later in toad muscle. 'Triggered' by the shock, with this unexplained delay, it develops its maximum rate at the start (like a condenser discharge). It is presumably the thermal sign of some chemical process by which the muscle is put, and maintained for an interval, in a state of 'activity', whatever that means, of readiness to shorten, to exert a force, or to do mechanical work.

Gasser & Hill (1924), working on a frog's sartorius at room temperature, showed that a sudden stretch applied even as early as 5 msec. after a shock found the muscle far less extensible than it was just before. This would be about 20 msec. at 0° C and the experiment ought to be repeated at 0° C with a muscle excited at a number of points to ensure approximate simultaneity of response all over; the expectation is that a change of extensibility would be detected as early as the heat. Brown (1934) found that the effect of a high pressure on the isometric twitch of a frog's muscle at 4.2° C was considerably to augment and slow it. If the pressure was released at the moment at which the shock was applied the augmentation disappeared, but if release took place very shortly after the shock a large part of the augmentation still occurred. In a later paper (1936) Brown showed that in the isometric twitch of the retractor penis of a tortoise the tension developed is much greater if a high pressure is applied before the shock, but little or no greater if the pressure is applied shortly after it; in a twitch reaching maximum tension (at 20° C) in about 0.2 sec. a compression begun 0.04 sec. after the shock had no augmenting effect. All such effects should, if possible, be examined at 0° C. They seem to agree with the heat measurements in showing that the greatest activity in a twitch occurs very soon after stimulation.

Von Muralt (1935) has given a very full account of various physical accompaniments of muscular activity. Not many of them can be recorded quickly enough to throw much light on the earlier stages of contraction. Von Muralt's own curves (1932) of the change of double refraction in the isometric contraction of a frog's striated muscle show a very rapid decrease at first, when the muscle (or at least its anisotropic bands) must have been shortening internally, but give no suggestion that optical changes do other than accompany mechanical ones. D. K. Hill (1949) has shown that a change of transparency begins in a frog's sartorius immediately at the end of the mechanical latent period, and that this is not due to change of length. Unfortunately (Hill 1948*a*), the rapid reversible changes of volume associated with contraction and relaxation, being due to changes of pressure caused by contraction, cannot be used to throw any new light on the onset of activity, and no other physical effects accompanying contraction can be recorded as yet with sufficient speed. For example, Dubuisson (1939) states that his glass electrode took about 4 sec. for an instantaneous variation of pH to be translated into a steady deflexion, and the considerations given in a recent paper (Hill 1948*b*) show that even if the glass electrode itself were instantaneous, diffusion from the inside of the muscle fibres to its surface is far too slow to allow rapid changes of pH to be accurately followed. Indeed, any method depending on diffusion to the outside of chemical substances produced within a muscle fibre cannot compare in speed with methods involving mechanical, thermal, optical, electrical or volume measurements. In the absence, therefore, of any present possibility of examining chemical changes of the very small magnitude and in the very short times involved,\* the

\* The total heat in a single twitch is about  $3 \times 10^{-3}$  cal./g. If the breakdown of ATP, or of creatine phosphate, were the source of this, about  $2.5 \times 10^{-7}$  g.mol. of phosphate would be set free per gram of muscle. To detect such a change at all would be a formidable task, to resolve its occurrence in times of the order of a few milliseconds would be one of fantastic difficulty.

chief hope of obtaining further knowledge of the onset and nature of contraction lies in improving and quickening the physical techniques which allow approximate simultaneity of recording.

There has been much speculation about the chemical nature of muscular contraction. The evidence on which this has been based has been chiefly derived of late from experiments on muscle extracts and enzymes. It is claimed, for example, that on the arrival of a nerve impulse ATP is broken down providing the energy for contraction; or, alternatively, that the combination of ATP with myosin is the immediate cause of shortening, relaxation occurring at the expense of free energy derived from the dephosphorylation of the ATP. According to Meyerhof, however (1947, p. 828), 'we should not...treat this hypothesis...that ATP breakdown initiates the chain of events in activity, as an established fact'; and he earlier expressed the opinion (1941, p. 240) 'these processes (breakdown and resynthesis of creatine phosphate and ATP) have primarily an energetic significance and are only one or two steps nearer to the development of the viscous elastic change in the contractile elements of the muscle than the breakdown of carbohydrate is'. In truth, the breakdown of ATP has never been observed in an intact muscle except in extreme fatigue verging on rigor.

The results of the investigations described in the present series of papers throw no direct light on the chemical problem. They do not even enable us to decide whether we should ascribe contraction to a liberation of free energy by some chemical breakdown, or, on the other hand, to an increase of entropy in the temporary 'randomization' of the molecular pattern. They do, however, provide us with the skeleton of the structure which has to be built up. It is useless, for example, to assert that contraction is an endothermic process (Szent-Györgyi 1947) when it is evidently exothermic throughout; nor can we usefully discuss the possibility that mechanical energy is first liberated from some latent store and chemical restorative processes then follow, in the light of such evidence as is contained in figures 3, 5 and 6 above. Again, if we wish to assume that energetic chemical processes produce relaxation we must accept the peculiar fact that their net heat change is precisely *nil*. Any chemical theory must take account of the liberation of energy in three separate forms, as heat of activation, as heat of shortening and as mechanical work.

One warning perhaps may be repeated. Owing to the extreme smallness and quickness of the chemical changes involved in a single twitch one naturally turns to the prolonged tetanic contraction which provides changes very many times greater. There is danger, however, in doing so uncritically. When a long-continued 'tetanus' of individual shocks is applied, at a frequency high enough to produce apparent fusion of the mechanical response, the resulting 'contraction' is not a simple elementary process as it is in a single twitch. The maintained contraction due to a tetanus is really the net result of the responses to all the shocks applied, their relaxation as well as their contraction. At any moment during a tetanus, contraction and relaxation are both occurring; the former predominates at the beginning, the latter at the end of the stimulus. Thus, chemical measurements made immediately after a tetanus cannot be referred only to 'contraction' as

distinct from 'relaxation', since both processes have been going on concurrently from very shortly after the start. Moreover, the early stages of recovery set in very soon and may already be going on fast while the muscle is still fully contracted. To assume that what happens during a tetanus, or in the next few seconds after it, corresponds simply to 'contraction', or 'relaxation', is more likely to lead to confusion than to understanding.

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# The haemoglobins of *Ascaris lumbricoides*

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The occurrence of distinct haemoglobins in the perienteric fluid and body wall of *Ascaris* is confirmed and methods described for the extraction and partial purification of the pigments.

The absorption spectra of the haemoglobins and their principal derivatives were determined.

Both haemoglobins possess a remarkable resistance to deoxygenation. This is due principally to a low deoxygenation velocity. In presence of  $\text{Na}_2\text{S}_2\text{O}_4$  at  $20.5^\circ\text{C}$ , pH 6,  $t_{50}$  for the deoxygenation of the perienteric fluid haemoglobin is 150 sec. compared with 0.008 sec. for sheep haemoglobin (Hartridge & Roughton 1923). With the body-wall haemoglobin the reaction is more rapid;  $t_{50}$  at pH 6,  $3^\circ\text{C}$ , is  $80 \pm 10$  sec.

The deoxygenation reaction is accurately unimolecular and is independent of the concentration of the reducing agent.

With perienteric fluid haemoglobin the temperature coefficient of the reaction is 5 and the velocity is increased with increase of pH between 5 and 9.

The velocity of conversion of the oxyhaemoglobins to methaemoglobin in presence of  $\text{K}_3\text{Fe}(\text{CN})_6$  was measured. *In vacuo*  $t_{50}$  is the same as for the deoxygenation reaction but diverges from unimolecular characteristics beyond half completion. Although the velocity is independent of the ferricyanide concentration, a measurable back reaction with  $\text{O}_2$  occurs.

$\text{CO}$  dissociates from both haemoglobins more rapidly than oxygen; 300 times more rapidly with perienteric fluid haemoglobin at pH 6,  $3^\circ\text{C}$ .

When *Ascaris* is kept under anaerobic conditions the body-wall haemoglobin becomes deoxygenated but no change could be detected in the perienteric fluid haemoglobin.

## INTRODUCTION

The nature of the metabolic process in nematodes parasitic within the alimentary canal of mammals has been the subject of controversy since Weinland (1901) claimed to have proved that *Ascaris lumbricoides* is an obligate anaerobe. A review of the progress of this controversy has been given by Laser (1944) together with additional evidence that the parasite is not completely independent of a supply of oxygen. He concluded that the oxidative enzymic system of *Ascaris* shows a perfect adaptation to low  $\text{O}_2$  tension; or conversely, that the low  $\text{O}_2$  requirement is conditioned by the peculiar enzymic system.

The unusual  $\text{O}_2$  relations of *Ascaris* lends interest to the observation of Keilin (1925) that the parasite possesses two spectroscopically distinct haemoglobins. The pigments occur respectively in the perienteric fluid and body wall of the worms. Von Brand (1938) was inclined to attribute to the haemoglobins a functional role as oxygen carriers.

The object of the present paper is the study of the properties of the *Ascaris* haemoglobins with particular reference to their possible function as oxygen carriers at extremely low tensions of the gas.

## EXPERIMENTAL

The source, method of collection, and manner of keeping the *Ascaris* material used in this work has been described by Baldwin (1943). Batches of worms always exhibit the pink coloration due to haemoglobin, but the intensity of this coloration shows wide individual variations.

Strips of the body wall, consisting of cuticle, hypodermis, and muscle, when viewed with the Hartridge spectrometer against a strong light, exhibit the spectrum of oxyhaemoglobin with the bands at  $\alpha = 5798 \text{ \AA}$ ;  $\beta = 5425 \text{ \AA}$ . Examination of the same preparation with the Zeiss microspectroscope as microscope ocular shows the spectrum to be most intense in the lateral field thickenings of the hypodermis. It is possible by careful scraping to separate first the muscle layer and then the hypodermis from the cuticle. Viewed in layers of similar thickness, the oxyhaemoglobin spectrum in the hypodermal tissue is about three times more intense than that of the muscle layer. In both tissues the position of the absorption bands is the same.

The absorption bands of the perienteric fluid oxyhaemoglobin are situated nearer to the blue than those of the body-wall pigment. The positions are  $\alpha = 5784 \text{ \AA}$ ,  $\beta = 5415 \text{ \AA}$ .

The washed gut of *Ascaris* contains only traces of haemoglobin but the constant presence of other haematin derivatives may be demonstrated by digesting the gut in  $\text{N KOH}$ , reducing with  $\text{Na}_2\text{S}_2\text{O}_4$ , and examining spectroscopically. The spectrum of haemochromogen, having the  $\alpha$ -band at  $5576 \text{ \AA}$ , is seen. The haematin content of the gut is usually low but worms were occasionally observed to have the organ coloured dark brown by aggregations of haematin. This condition was always correlated with abnormally high concentrations of haemoglobin in both the body wall and the perienteric fluid.

*Extraction of the haemoglobins*

An average sample of *Ascaris* perienteric fluid, from worms kept for 18 hr. in the balanced saline medium described by Baldwin (1943), has a dry weight of  $60.0 \text{ mg./ml.}$  About half the dry matter is protein ( $32.5 \text{ mg./ml.}$ ) The haematin content is usually  $3.3 \times 10^{-5} \text{ M}$ . Assuming  $1 \text{ g.mol.}$  haematin to be equivalent to  $17,000 \text{ g.}$  haemoglobin, then the haemoglobin of the perienteric fluid represents only 2% of the total protein.

The fluid was removed by slitting the worms and draining in a funnel. Dialysis against distilled water in cellophane for 24 hr. gave a dense precipitate which was removed by centrifuging. The supernatant was brought by dialysis to 0.53 saturation  $(\text{NH}_4)_2\text{SO}_4$  at pH 7 and the precipitated protein centrifuged off and rejected. The bright red solution was then dialyzed against  $(\text{NH}_4)_2\text{SO}_4$  to 0.73 saturation. The precipitate, which contained all the haemoglobin, was separated in the centrifuge, dissolved in the minimum phosphate buffer pH 7 and dialyzed against distilled water. This solution contained usually  $2.3 \times 10^{-4} \text{ M}$  haematin, representing as haemoglobin 12% of the dry weight.

Solutions having a haemoglobin content of 20% of the dry weight could be obtained by further fractionation but, in view of the small amount of material

available, the losses attending the fractionation, and the tendency of the pigment to form methaemoglobin, the less pure solution was used in the experiment is to be described.

After removal of the perienteric fluid, the integument of the worms was freed from gut and reproductive organs and extracted overnight with distilled water. The pink solution was fractionated in the same way as the perienteric fluid, with the omission of the preliminary dialysis. The final solution usually contained  $2.5 \times 10^{-4}$  M haematin, representing, as haemoglobin, 12 to 15 % of the dry weight.

#### *Estimation of haemoglobin*

Haemoglobin was estimated as haematin by measuring the light absorption in the  $\alpha$ -band of the haemochromogen  $p$  produced by simultaneous denaturation and reduction. The suitably diluted haemoglobin solution was added, in the cell of the Hilger-Nutting spectrophotometer, to an equal volume of a solution of  $\text{Na}_2\text{S}_2\text{O}_4$  in N NaOH. The density at  $5576 \text{ \AA}$  was read 10 min. after mixing.

Haemin, prepared and recrystallized by the method of Fischer (1922), was used as standard. For this purpose a solution of alkaline haematin was prepared by dissolving a weighed amount of haemin in 0.5 N NaOH. *Ascaris* protein, spectroscopically free from haematin, was prepared by treating a concentrated *Ascaris* haemoglobin solution with acid acetone. A 0.4 % solution of the protein was made up in 0.5 N NaOH. From these solutions the standard haemochromogen was developed in the spectrophotometer cell by adding 1 vol. of the suitably diluted haematin solution to 2 vol. of the globin solution to which excess of  $\text{Na}_2\text{S}_2\text{O}_4$  had been added. Ten minutes after mixing the density at  $5576 \text{ \AA}$  was read. Absorption coefficients  $\beta$  were calculated from the formula

$$\beta = \frac{1}{cd} \log_e \frac{I_0}{I},$$

where  $c$  is the concentration of haematin in g.mol./ml.,  $d$  is the thickness of the absorbing layer,  $I_0$  and  $I$  are the intensities of incident and transmitted light respectively. For the haemochromogen standard  $\beta_{5576} = 0.64 \times 10^8$ .

When pig globin was substituted for *Ascaris* protein in the preparation of the haemochromogen standard the value of  $\beta$  was unchanged but the  $\alpha$ -band maximum was situated nearer the red at  $5585 \text{ \AA}$ . The observation that the position of the  $\alpha$ -band in haemochromogen prepared directly from *Ascaris* haemoglobin is the same as in the haemochromogen prepared from haemin and *Ascaris* protein, is evidence that the haemoglobins have ferroprotoporphyrin as prosthetic group.

#### THE PROPERTIES OF THE HAEMOGLOBINS

##### *Absorption spectra of the oxyhaemoglobins*

The absorption spectra of the oxyhaemoglobins were measured in a Hilger-Nutting visual spectrophotometer. Apart from the different positions of the absorption maxima, the results for both oxyhaemoglobins were similar and in both pigments absorption in the  $\beta$ -band is greater than in the  $\alpha$ -band. This condition is

not typical of oxyhaemoglobin from other animal sources where the  $\alpha$ -band slightly exceeds the  $\beta$ -band in intensity. Keilin & Wang (1945) have, however, described a similar type of spectrum in the oxyhaemoglobin they isolated from the nodules of leguminous roots.

*The dissociation of oxygen from the oxyhaemoglobins*

The essential property of haemoglobin is the capacity to enter into a labile combination with molecular oxygen and to give up this oxygen at low tensions of the gas. Both *Ascaris* oxyhaemoglobins were found to possess an exceptional resistance to deoxygenation when they were equilibrated against vacuum. No change could be detected in the spectrum of a solution of the perienteric fluid oxyhaemoglobin, pH 7 after equilibration against a frequently renewed vacuum in a Thunberg tube at 20°C for 1 hr. After 3 hr., evidence of slight deoxygenation was given by an increase in the intensity of the oxyhaemoglobin spectrum when air was readmitted to the tube.

Deoxygenation of the body-wall oxyhaemoglobin occurred more readily. In 3 hr. at 20°C about half the pigment was in the deoxygenated form.

Prolonged exposure to low tensions of oxygen, under these conditions, hastened the formation of methaemoglobin and even gentle shaking resulted in some denaturation of the pigment. It was therefore not found possible to secure complete deoxygenation of either pigment by extending the duration of the equilibration. Thus the usual methods for determining the oxygen equilibrium curves could not be applied to the *Ascaris* haemoglobins. Nevertheless the results are a qualitative indication that the oxygen affinities of the *Ascaris* haemoglobins are unusually high. Under the same conditions of equilibration it was possible to deoxygenate horse-muscle haemoglobin completely in 5 min.

*The kinetics of the deoxygenation reaction*

When  $\text{Na}_2\text{S}_2\text{O}_4$  is added to the *Ascaris* oxyhaemoglobins the two absorption bands are replaced by a single band at  $555\ \mu$ . The spectrum (figure 1) is that of a typical haemoglobin. The remarkable feature of the change is that it occurs slowly, in contrast to the apparently instantaneous deoxygenation of other haemoglobins in presence of this reducer. Subsequent reoxygenation completely restores the spectrum of oxyhaemoglobin.

The velocity with which oxygen dissociates from a number of vertebrate haemoglobins, in presence of  $\text{Na}_2\text{S}_2\text{O}_4$ , was investigated by Hartridge & Roughton (1923) and by Millikan (1933, 1936). Salomon (1941) made a similar study of two invertebrate haemoglobins. These reactions proceeded so rapidly that the ingenious rapid-flow technique devised by Hartridge & Roughton (1923) was necessary for their measurement. The slowness of the reaction with the *Ascaris* haemoglobins made possible the use of a simple static method.

*Method*

*Ascaris* oxyhaemoglobin, in an evacuated Thunberg tube, was rapidly mixed with  $\text{Na}_2\text{S}_2\text{O}_4$ . The progress of the reaction was followed by comparing the spectrum



of the solution in the tube with that of a variable optical mixture of haemoglobin and oxyhaemoglobin in the spectrophotometer arrangement described by Hill (1936).

Each Thunberg tube contained 3 ml. of a dilute solution of oxyhaemoglobin (ca.  $0.7 \times 10^{-4}$  M haematin) in M/15 buffer. The required amount of solid  $\text{Na}_2\text{S}_2\text{O}_4$  was weighed into the hollow stopper. After evacuation of the tube the reaction was started by inverting the tube and shaking to ensure rapid solution of the reducer. The cups of the spectrophotometer contained, respectively, *Ascaris* oxyhaemoglobin and haemoglobin diluted to the same effective concentration as the

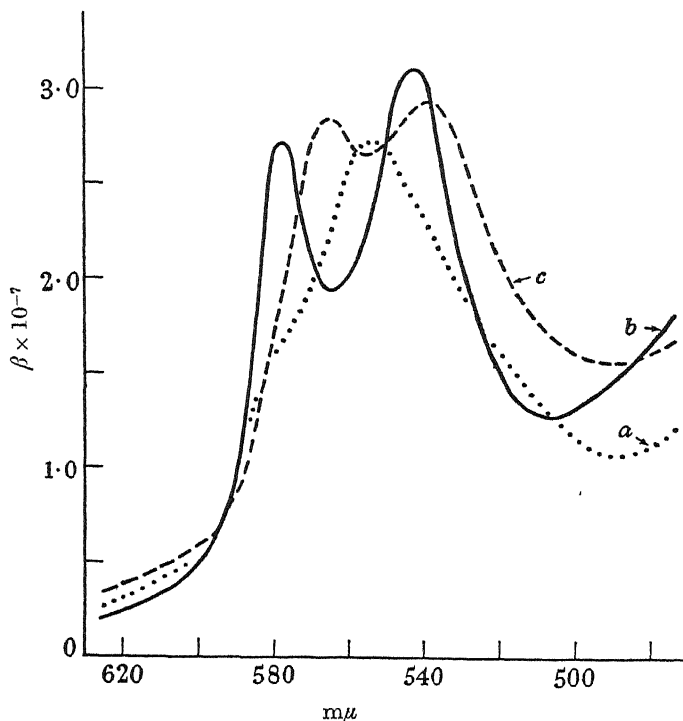


FIGURE 1. Absorption spectra of *Ascaris* body-wall haemoglobin (a), oxyhaemoglobin (b) and CO-haemoglobin (c).

pigment in the vacuum tube. The haemoglobin solution contained excess  $\text{Na}_2\text{S}_2\text{O}_4$  and a layer of liquid paraffin above the solution retarded the ingress of air.

During the reaction the vacuum tube was held upright in a glass water bath between the upper light source and the microspectroscope. The temperature of the water in the bath was thermostatically controlled.

After the reaction commenced readings were taken at convenient intervals until deoxygenation was about 85 % complete.

In each series of experiments the haemoglobin in the reaction tubes and in the spectrophotometer cups was derived from a single preparation of the pigment. Occasional preparations contained up to 10 % methaemoglobin. At the start of the reaction the methaemoglobin fraction in the vacuum tube was immediately reduced to haemoglobin. Since one cup of the colorimeter still contained a mixture

of oxyhaemoglobin and methaemoglobin, accurate matching of the spectra was impossible. To overcome this difficulty the methaemoglobin in the cup was converted to oxyhaemoglobin by the addition of ferrous potassium oxalate.

## RESULTS

### *Perienteric fluid haemoglobin*

The results of a typical experiment with the perienteric fluid haemoglobin are given in figure 2*a*. The data has been replotted on a semilogarithmic scale to show that the reaction follows a strictly unimolecular course over the entire series of readings (figure 2*b*). The result is analogous to those obtained by Hartridge & Roughton (1923) with sheep haemoglobin but, although the order of the reaction is the same, the magnitude of the time scale is very different. Thus the time for half completion ( $t_{50}$ ) of the reaction shown in figure 2 is 150.0 sec. compared with 0.008 sec. for sheep haemoglobin under the same conditions.

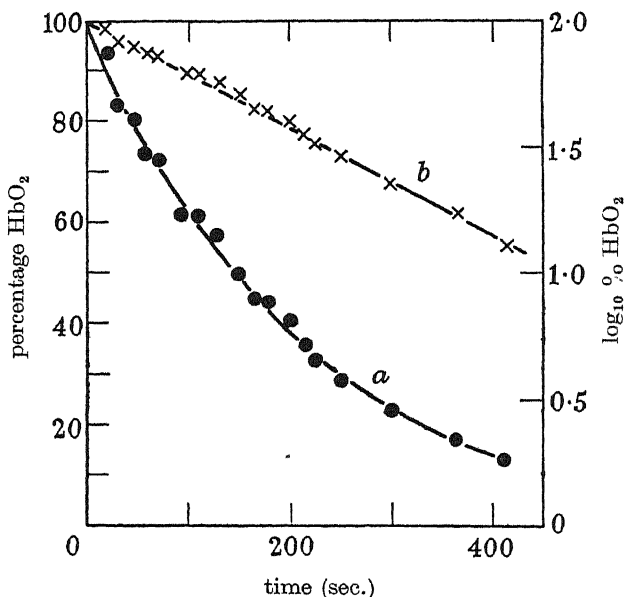


FIGURE 2. Dissociation of oxygen from combination with *Ascaris* perienteric fluid haemoglobin. pH 6, 20.5°C. *In vacuo*. Ordinates for *a* on left and for *b* on right.

### *The effect of varying the concentration of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>*

In a series of measurements carried out under otherwise similar conditions, the concentration of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> was varied eightfold. The results of these experiments (table 1) showed that this variation was without significant effect upon the reaction velocity.

From this it was inferred that if any direct reduction of the oxyhaemoglobin molecule occurs, it plays an insignificant part in the overall reaction. Similar results with sheep haemoglobin led Hartridge & Roughton (1923) to conclude that Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> reacts only with oxygen liberated by the dissociation of oxyhaemoglobin,

removing this oxygen faster than it recombine with haemoglobin, thus reducing the back reaction to zero. This interpretation of the mechanism of the reaction can therefore be extended to the *Ascaris* pigment, confirmation that the pigment possesses the capacity to undergo reversible oxygenation, the essential property of haemoglobin.

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF  $\text{Na}_2\text{S}_2\text{O}_4$  ON THE DEOXYGENATION VELOCITY OF *ASCARIS* PERIENTERIC FLUID HAEMOGLOBIN IN *VACUO*

Haemoglobin = haematin =  $0.6 \times 10^{-4}$  M pH 7,  $21.5^\circ\text{C}$

$t_{50}$ (sec.)	percentage $\text{Na}_2\text{S}_2\text{O}_4$	$k \times 10^4$
140.0	0.04	50.0
160.0	0.133	43.8
150.0	0.20	46.7
130.0	0.33	53.8

### The effect of pH

The change from acid to alkaline pH, within the physiological range, is accompanied by an increase in the deoxygenation velocity. The results of the determinations are shown as a graph in figure 3 where the dissociation velocity constant is plotted against pH. The values of  $k$  are given by the formula  $k = \log_e 2/t_{50}$  derived from the equation for a unimolecular reaction. Below pH 6 the reaction velocity remains steady but, with increasing alkalinity to pH 9 the value of  $t_{50}$  diminishes from 400 to 200 sec. Difficulty was experienced, at pH's more alkaline than 9, in preventing the denaturation of some haemoglobin. Where denaturation had occurred an intense haemochromogen band appeared immediately the reaction was commenced. When this was observed no further readings were taken.

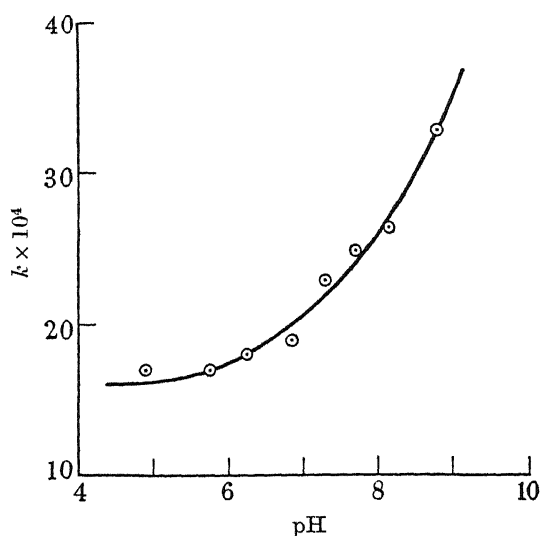


FIGURE 3. Influence of pH upon deoxygenation velocity of *Ascaris* perienteric fluid haemoglobin.  $16^\circ\text{C}$ .

In order to verify that the pH effect was not due to a change in the mode of action of the reducing agent with increasing pH, the concentration of  $\text{Na}_2\text{S}_2\text{O}_4$  was varied ninefold in a series of determinations carried out at pH 8.5. The variation was without effect upon the reaction velocity, thus excluding the possibility that there was a direct reduction of oxyhaemoglobin at the higher pH values.

The sensitivity of the oxygen equilibrium curves of vertebrate haemoglobins to changes in pH was shown by Hartridge & Roughton (1923) to be due to the effect of this factor upon the deoxygenation velocity. With sheep haemoglobin deoxygenation at pH 5 was about eight times faster than at pH 8. Compared with mammalian haemoglobin, the effect of pH on the dissociation of oxygen from *Ascaris* perienteric fluid haemoglobin is thus reversed in direction and smaller in magnitude.

#### *The effect of temperature*

Although *Ascaris* is a poikilothermous animal, incapable of temperature regulation, its habitat within the gut of a mammal ensures the maintenance of a constant temperature around  $38^\circ\text{C}$ . The effect of temperature variation upon the deoxygenation velocity of the haemoglobin was therefore examined. Figure 4 expresses the results of measurements at temperatures between  $4^\circ$  and  $20^\circ\text{C}$ . Over this range the reaction velocity increases more than twenty times.

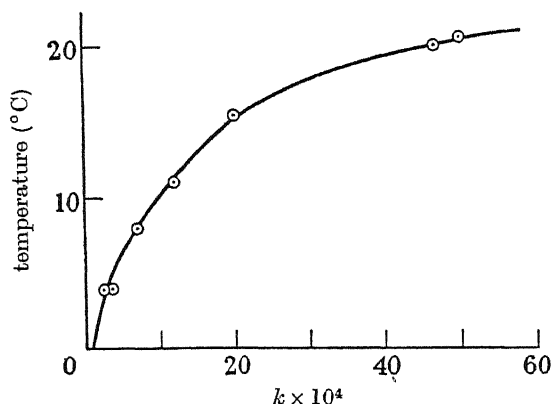


FIGURE 4. Influence of temperature upon the deoxygenation velocity of *Ascaris* perienteric fluid haemoglobin, pH 6.5.

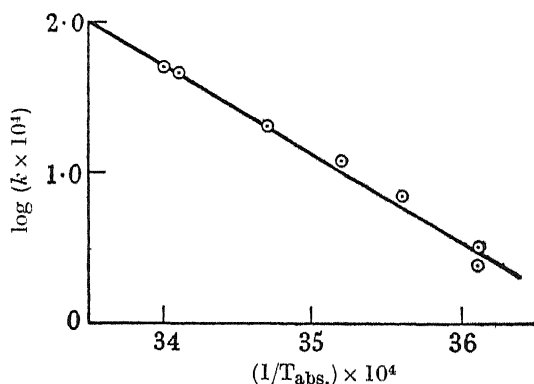


FIGURE 5. Influence of temperature upon the deoxygenation velocity of *Ascaris* perienteric fluid haemoglobin. Data from figure 4.

It follows from the Arrhenius equation that  $\log k$  is proportional to  $1/T$ , where  $T$  is the absolute temperature. The data are plotted in figure 5 to show this relationship. The reaction has the high temperature coefficient of 5, compared with the value of 3.8 obtained by Hartridge & Roughton (1923) for sheep haemoglobin. From figure 5 it is possible to extrapolate to  $38^\circ\text{C}$ , giving 10 sec. as the approximate value of  $t_{50}$  at that temperature. By a similar extrapolation Hartridge & Roughton deduced that  $t_{50}$  for sheep haemoglobin at  $37^\circ\text{C}$  would be 0.0025 sec. Although the temperature coefficient of the reaction with *Ascaris* haemoglobin is high, it is not sufficiently great to give a reaction velocity at mammalian body temperature comparable with that of mammalian haemoglobin.

*The body-wall haemoglobin*

Preliminary observations showed that *Ascaris* body-wall oxyhaemoglobin is deoxygenated more rapidly than the perienteric fluid pigment and in order that sufficient readings could be taken for the characteristics of the reaction to be examined, the measurements were carried out at 3°C. With fresh preparations of the oxyhaemoglobin, 60 hr. after the worms were killed, the value of  $t_{50}$  in measurements at pH 6, 3°C is  $80 \pm 5$  sec., compared with 2300 sec. for perienteric fluid haemoglobin under the same conditions. The reaction is accurately unimolecular and its velocity independent of the concentration of the reducer.

Consecutive determinations of the deoxygenation velocity of any single preparation of the haemoglobin gave consistent results, but wide variations were often obtained in the results from different preparations. These variations were found to be the result of a slow diminution in the deoxygenation velocity with time, but preparations up to one week old were always deoxygenated at least four times faster than the perienteric fluid haemoglobin under the same conditions.

*The formation of methaemoglobin*

Spontaneous oxidation of the *Ascaris* oxyhaemoglobins to methaemoglobin occurs extremely slowly. In presence of  $K_3Fe(CN)_6$  at pH 6, acid methaemoglobins are formed. The spectrum is normal with major bands at 500 and 630 m $\mu$ . The alkaline methaemoglobins, formed at pH 9, have an unusual spectrum dominated by a strong but diffuse band at 537 m $\mu$  with a very faint  $\alpha$ -band at 565 m $\mu$ . This spectrum is in striking contrast to that of mammalian alkaline methaemoglobin, where the bands are distinct and differ little in intensity.

The partition between the alkaline and acid forms of the perienteric fluid methaemoglobin was measured by comparing the spectra of solutions at different pH's with a variable optical mixture of the acid and alkaline forms in the cups of the spectrophotometer. The pK of the *Ascaris* methaemoglobin is 7.4, compared with 8.2 for pig methaemoglobin.

*The velocity of formation of methaemoglobin*

When  $K_3Fe(CN)_6$  is used to oxidize the oxyhaemoglobins to methaemoglobin the change occurs slowly. This observation was not unexpected since, according to Conant (1923), ferricyanide reacts only with dissociated haemoglobin and not directly with oxyhaemoglobin. Assuming the validity of this conclusion, and in the absence of a back reaction, the velocity of oxidation becomes a measure of the deoxygenation velocity. Measurements were therefore made of the rate of formation of methaemoglobin from the *Ascaris* oxyhaemoglobins in presence of ferricyanide in order that the reaction could be compared with the deoxygenation reaction in presence of  $Na_2S_2O_4$ .

*Method*

The method was similar to that used in the deoxygenation velocity measurements. In the stopper of the vacuum tube solid  $K_3Fe(CN)_6$  replaced  $Na_2S_2O_4$  and the cups

of the spectrocoulometer contained methaemoglobin (pH 6) and oxyhaemoglobin respectively. All experiments were carried out at pH 6, where the contrast between the spectra of the two components of the reaction is greatest.

### Results

The data for a typical experiment with the body-wall haemoglobin *in vacuo* is plotted on a semilogarithmic scale in figure 6. In the same figure the result of a deoxygenation experiment on the same preparation under the same conditions is also shown. The initial velocity of the two reactions is the same, giving identical values of  $t_{50}$ . In its later stages however, the velocity of the ferricyanide reaction diminishes and the semilogarithmic points diverge from the straight line characteristic of a unimolecular reaction.

Similar results were obtained with the perienteric fluid haemoglobin, but here the departure from unimolecular characteristics is smaller. Neither the initial velocity of the reaction, nor its subsequent course, were influenced by a fourteenfold variation in the ferricyanide concentration. Direct reaction between oxyhaemoglobin and ferricyanide does not therefore occur.

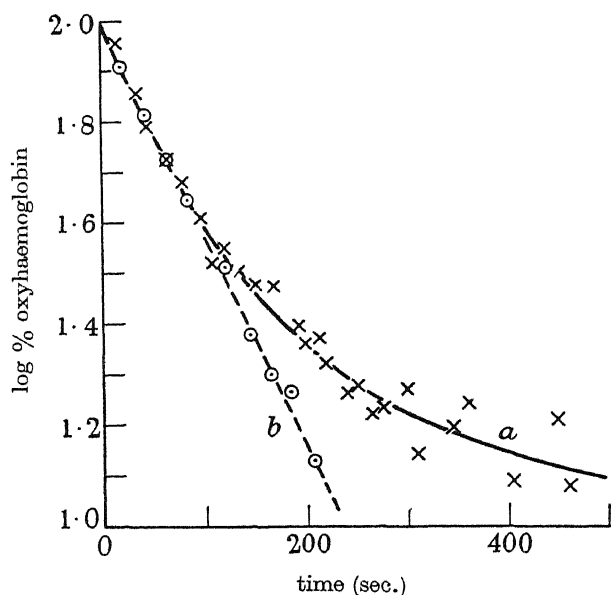


FIGURE 6. Comparison of the reaction of *Ascaris* body-wall oxyhaemoglobin, *in vacuo*, in presence of: (a)  $\text{K}_3\text{Fe}(\text{CN})_6 = 3 \times 10^{-3} \text{ M}$ , (b)  $\text{Na}_2\text{S}_2\text{O}_4$ , pH 6,  $3.5^\circ \text{C}$ .

Where experiments are carried out *in vacuo*, the initial oxygen tension is negligible, but oxygen liberated in the reaction accumulates to a final concentration equal to that of the haemoglobin haematin. It was conceivable that the departure from unimolecular characteristics was due to a back reaction between this oxygen and dissociated haemoglobin. Control experiments with oxyhaemoglobin which had been equilibrated against air or oxygen showed that the initial velocity of the reaction was decreased, proving the existence of a back reaction at high oxygen

tensions. Table 2 gives the results of these experiments with perienteric fluid haemoglobin. The magnitude of the effect is small. A fivefold increase in oxygen tension between air and oxygen results in a 16 % diminution in the reaction velocity. The effect of the small amount of oxygen liberated from the low concentration of oxyhaemoglobin used in experiments *in vacuo* would therefore lie well within the experimental error of the method of measurement. It was concluded that the divergence from the characteristics of a unimolecular reaction could not be explained as the result of a back reaction.

TABLE 2. THE EFFECT OF VARIATIONS IN THE  $O_2$  TENSION UPON THE REACTION VELOCITY OF *ASCARIS* PERIENTERIC FLUID OXYHAEMOGLOBIN IN PRESENCE OF FERRICYANIDE

Haematin =  $0.7 \times 10^{-4}$  M.  $K_3Fe(CN)_6$  =  $19.0 \times 10^{-3}$  M.  $12^\circ C$ . pH 6, Bar. = 756 mm. Hg

	$O_2$ concentration at $t_{50}$	$t_{50}$ (sec.)	percentage HbO <sub>2</sub> at 800 sec.
vacuum	$0.35 \times 10^{-4}$	$800 \pm 50$	50
air	$3.55 \times 10^{-4}$	$1050 \pm 50$	59
oxygen	$15.35 \times 10^{-4}$	$1250 \pm 50$	64

Roughton (1934) suggested that nothing is known of the effect of oxidizing one or more of the iron atoms in mammalian CO-haemoglobin, upon the dissociation of CO from another of the four iron atoms. The number of haem groups in the *Ascaris* haemoglobin molecule is not known, but the assumption that oxidation of one iron atom diminishes the tendency of oxygen to dissociate from other haem groups in the molecule would provide a possible explanation of the divergence of the ferricyanide reaction from unimolecular characteristics. The molecule of mammalian muscle haemoglobin contains one haem and Millikan (1936) found dissociation of CO from this haemoglobin, in presence of ferricyanide, to be strictly unimolecular.

#### *Deoxygenation of the haemoglobins in vivo*

In view of the remarkable resistance shown by the *Ascaris* haemoglobins to deoxygenation, experiments were carried out to determine whether the worm is capable of bringing about deoxygenation *in vivo*.

In these experiments, six large female *Ascaris* were confined in a tightly stoppered 150 ml. flask containing normal saline from which all air had been removed by boiling. Alternatively, individual worms were corked up in narrow tubes containing saline. The tubes or flasks were incubated at  $37^\circ C$  and examined spectroscopically against a strong light at intervals.

Signs of deoxygenation were observed in about 2 hr. when the bands of oxyhaemoglobin began to diminish in intensity and the colour of the worms became duller. At this stage the worms became inactive. After 6 hr. incubation the bands of oxyhaemoglobin were very faint. Continued incubation resulted in no further change but the oxyhaemoglobin spectrum was fully restored when air was again admitted.

There was evidence that only the body-wall oxyhaemoglobin underwent deoxygenation and that the faint oxyhaemoglobin spectrum which always persisted after prolonged anaerobiosis, was due to the perienteric oxyhaemoglobin. The portion of a female *Ascaris* anterior to the genital pore is very muscular, with a greatly constricted perienteric cavity. Behind the genital pore the reproductive organs lie in a much dilated perienteric space and the body wall is relatively thinner. The perienteric fluid haemoglobin thus contributes proportionately more to the spectrum of the posterior region of the worm than to the anterior region. In worms which had been incubated in tubes for 6 hr. the residual oxyhaemoglobin spectrum was relatively stronger in the posterior region.

Confirmation that the perienteric fluid haemoglobin resists deoxygenation under these conditions was obtained by withdrawing the fluid with precautions to prevent the ingress of air. The dead space of a record syringe was filled with medicinal paraffin and perienteric fluid extracted from worms incubated for 6 hr. Spectroscopic examination of the fluid through the glass barrel of the syringe gave no indication that any deoxygenation had occurred.

#### *Relations of the haemoglobins with CO*

When CO was passed through a solution of either of the *Ascaris* haemoglobins, previously deoxygenated with  $\text{Na}_2\text{S}_2\text{O}_4$  the solution immediately became cherry red and showed the spectrum of CO-haemoglobin (figure 1). Although the absorption bands are more diffuse, they resemble, both in their relative and absolute intensities, the bands of mammalian CO-haemoglobin. The 'span' (distance separating the bands of  $\text{HbO}_2$  and  $\text{HbCO}$ ) of both *Ascaris* haemoglobins is 90 to 95 Å. This high value they share with the haemoglobin of leguminous root nodules (Keilin & Wang 1945) and that of *Gastrophilus* larvae (Keilin & Wang 1946) where the spans are 100 Å and 95 Å respectively.

Carbon monoxide displaces oxygen from combination with the *Ascaris* haemoglobins extremely slowly. Equilibration against pure CO for 1 hr. at 20°C resulted in a shift of the band of 60 Å for the perienteric fluid and 80 Å for the body-wall haemoglobin. Prolonged equilibration with frequent renewal of CO is necessary for complete conversion. Moreover the CO-haemoglobins in the absence of a reducing agent rapidly revert to oxyhaemoglobin on exposure to air. Compared with the oxygen affinity the CO-affinity of the haemoglobins is therefore low.

#### *Velocity of dissociation of CO from the Ascaris haemoglobins*

In the absence of a suitable reagent for absorbing CO, Millikan (1936) measured the dissociation velocity of mammalian muscle CO-haemoglobin by using ferri-cyanide to oxidize dissociated haemoglobin as fast as it was produced. This method was applied to the *Ascaris* haemoglobin.

5 ml. of a solution of oxyhaemoglobin, pH 6, in a Thunberg tube were reduced with the minimum amount of  $\text{Na}_2\text{S}_2\text{O}_4$ . The hollow stopper, containing 40 mg.  $\text{K}_3\text{Fe}(\text{CN})_6$ , was replaced and the tube evacuated. Pure CO was then admitted to convert the haemoglobin to CO-haemoglobin. The reaction was started by rapidly



inverting the tube with shaking to ensure solution of the ferricyanide. Where possible the progress of the reaction was followed by comparing the spectrum of the solution in the tube with that of an optical mixture of methaemoglobin (pH 6) and CO-haemoglobin in the cups of the spectrophotometer. In order to diminish the effect due to the photochemical decomposition of carboxyhaemoglobin the illumination of the reaction tube was switched on only when a reading was being taken and was reduced to the minimum compatible with ease in comparing the spectra.

At 15°C the reaction with both haemoglobins was too rapid for measurement. At 3°C the rate was sufficiently diminished for two readings with each tube containing perienteric fluid haemoglobin but readings were still not possible with the body-wall pigment.

Figure 7 summarizes the results of three experiments with perienteric fluid haemoglobin plotted semilogarithmically. At 3°C,  $t_{50}$  was 10 sec. and  $k=0.07$ . Under similar conditions for the deoxygenation reaction  $k=2.5 \times 10^{-4}$ . Carbon monoxide thus dissociated from combination with the haemoglobin about 300 times faster than oxygen.

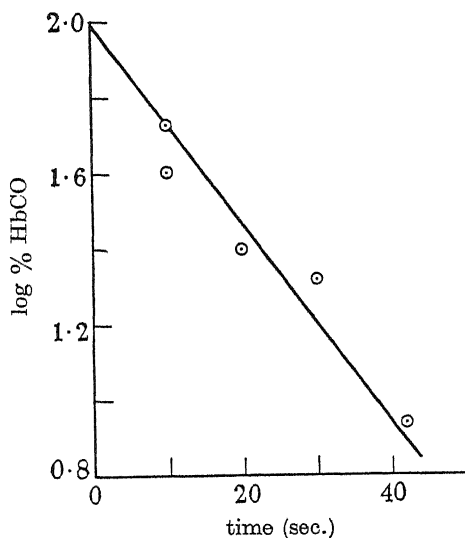


FIGURE 7. Reaction of *Ascaris* perienteric fluid carboxyhaemoglobin in presence of ferricyanide.  $K_3Fe(CN)_6 = 3 \times 10^{-4}$  M, pH 6, 3°C.

#### DISCUSSION

Most of our knowledge of the properties of haemoglobins, and the manner in which these properties are related to the oxygen requirements of the organism, has been derived from the study of vertebrate haemoglobins. In this phylum haemoglobin occurs universally and must have arisen at an early stage in evolution.

Among invertebrates, on the other hand, the distribution of the pigment is irregular. It is absent from some large phyla and in others where it occurs sporadically it may be absent or replaced by other respiratory pigments in closely related

forms. Keilin & Wang (1945, 1946) have pointed out that since all cells of aerobic organisms contain haematin catalysts every such cell is a carrier of the prosthetic group of haemoglobin. They suggest that the factor limiting the distribution of haemoglobin is the inability of many cells to synthesize the highly specific proteins which, combined with haem, give haemoglobin its essential property of reversible oxygenation.

The phylogenetic relationships of invertebrates possessing haemoglobin give no indication whether this ability has arisen *de novo* many times in evolution or whether the ability has been retained only in those forms where possession of haemoglobin confers some survival value. Svedberg & Eriksson (1933) considered that invertebrate haemoglobins formed, in their chemical properties, a homogeneous group distinct from vertebrate haemoglobins, and advocated a revival of the name erythrocrucorin to describe them. But Keilin & Wang (1945, 1946) in their studies of the haemoglobins of leguminous root nodules and *Gastrophilus* larvae have now shown that haemoglobins from non-vertebrate sources may differ as widely from the erythrocrucorins of Svedberg & Eriksson as from vertebrate haemoglobin. This diversity, associated with the discontinuous distribution, is strong evidence that the ability to synthesize haemoglobin arose independently a number of times in invertebrate evolution.

The properties of the *Ascaris* haemoglobins diverge still further from those of other haemoglobins. Most striking is the impossibility of bringing about deoxygenation in the absence of reducing agents, within a convenient time. It is of interest that Aducco (1889) extracted a haemoglobin from the nematode *Dioclophyme renale*, a kidney parasite of the dog, and observed a similar resistance to deoxygenation *in vacuo*.

The kinetic background to the oxygen equilibrium state in mammalian haemoglobins has been elucidated by Hartridge & Roughton (1923, 1925), Roughton (1934) and by Millikan (1933, 1936). The haemoglobins upon which they worked combined with, and dissociated from oxygen with great rapidity, but specific differences in their reaction velocities were detected. Millikan (1936) showed that the high oxygen affinity of mammalian muscle haemoglobin was the result of a high association velocity, the dissociation velocity being of the same order of magnitude as that of the blood of the same mammal. On the other hand, changes in the oxygen affinity arising with changes of pH or temperature in blood haemoglobin were shown by Hartridge & Roughton (1923) to be due to the influence of these factors upon the deoxygenation velocity.

Little work of this kind has been carried out using invertebrate haemoglobin. Salomon (1941) compared the deoxygenation velocities of the haemoglobins of two annelid worms and found that, although specific differences could again be detected, the reaction time for both pigments was of the same order of magnitude as for vertebrate haemoglobins. Table 3 illustrates the contrast between these results and the deoxygenation velocities of the *Ascaris* haemoglobins.

In spite of this contrast, the reaction of the *Ascaris* pigments, in presence of  $\text{Na}_2\text{S}_2\text{O}_4$ , has the characteristics of a deoxygenation and not a true reduction. The reaction is accurately unimolecular and its velocity independent of the concentra-

tion of the reducing agent. Moreover, oxidation of the oxyhaemoglobins in presence of ferricyanide proceeds initially at the same rate as the deoxygenation reaction.

Slow reactions of this kind are not without precedent in the gas relations of other haemoglobins. Roughton (1934) found that the velocity constant for dissociation of CO from sheep haemoglobin at pH 7.4 and 20°C is 0.044. Under the same conditions the same value was obtained for the deoxygenation reaction with *Ascaris* perienteric fluid haemoglobin.

TABLE 3

source of haemoglobin	$t_{50}$ deoxygenation (sec.)	pH	temperature (°C)	reference
Vertebrates				
frog	0.02	8.6	22	Millikan (1933)
sheep	0.028			
man	0.038			
pig	0.047			
horse (muscle)	0.02	8.6	20	Millikan (1936)
Invertebrates				
<i>Lumbricus</i>	0.07	8.0	23	Salomon (1941)
<i>Glycera</i>	0.027	8.52	28	
<i>Ascaris</i> (perienteric fluid)	440.0	5.5	16	this paper
	220.0	9.0	16	

Carbon monoxide dissociates from sheep haemoglobin 10,000 times more slowly than oxygen and associates only 30 times more slowly. The great affinity of sheep haemoglobin for CO, 250 times the affinity for oxygen is thus principally due to the low velocity of dissociation of CO from the pigment. The high oxygen affinities of the *Ascaris* haemoglobins are similarly associated with a low oxygen dissociation velocity.

Increase of pH within the range 5 to 9 increases the tendency for oxygen to dissociate from the perienteric fluid haemoglobin. Hartridge & Roughton (1923) found this pH effect to be in the reverse direction with sheep haemoglobin. Moreover, they observed that the curve relating pH to the dissociation constant resembles the ionisation curve of a weak monovalent acid, and that the effect accounted for the Bohr effect in the equilibrium curves of the haemoglobin. Ferry & Green (1929) observed a reversal of the Bohr effect below pH 6.5 and their findings have been correlated by German & Wyman (1937) with the base-binding power of haemoglobin and oxyhaemoglobin at different pH's. Under physiological conditions oxyhaemoglobin is a stronger acid than haemoglobin but German & Wyman found the reverse to be true between pH 4.5 and 6.1. In this acid range the effect of oxygenation is therefore to reduce the acid dissociation of one or more oxylabile groups.

In spite of a high temperature coefficient the low deoxygenation velocity of the perienteric fluid haemoglobin renders it ill fitted to serve in the rapid transference of oxygen to the oxidative enzyme system, and there is no evidence that deoxygenation occurs *in vivo*. All nematodes lack a blood vascular system and entry of

oxygen in those parasites which do not habitually ingest arterial blood of the host is by diffusion across the cuticle. In *Ascaris*, body-wall haemoglobin occurs in highest concentration in the hypodermal layer immediately below the cuticle. Although the haemoglobin has a very high oxygen affinity the deoxygenation velocity of fresh preparations, at the low temperatures where measurement by a static method was possible, is 40 times greater than in the perienteric fluid pigment. Compared with mammalian haemoglobin the reaction is still slow. Nevertheless *Ascaris* can utilize the oxygen bound by the body-wall haemoglobin and it is of interest that, at the point where deoxygenation commences, the worm becomes inactive. Slater (1925) found that when this inactivity was prevented by the application of induction shocks, the survival time of the worms was greatly diminished and he concluded that a supply of oxygen was essential for normal activity. This was the first direct evidence that *Ascaris* is not a complete anaerobe and led to numerous measurements of the oxygen uptake. Laser (1944) has reviewed this work and has shown that the oxidative enzyme system of the parasite is adapted to, or conditioned by, low oxygen tensions. It is probable that the body-wall haemoglobin plays a part in supplying the low oxygen requirement.

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# The haemoglobins of *Nippostrongylus muris* (Yokagawa) and *Strongylus* spp.

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## INTRODUCTION

From time to time, in connexion with work on the metabolism of parasitic nematodes, attention has been drawn to the presence of haemoglobin in a number of species. Aducco (1889) observed that the red colour of *Diectophyme renale* is due to a pigment resembling vertebrate haemoglobin. More recently haemoglobin has been recorded in *Ascaris lumbricoides* and *Parascaris equorum* (Keilin 1925), in *Nematodirus*, *Ostertagia* and other *Trichostrongylidae* of sheep (Davey 1938) and in *Camallanus* (Wharton 1938). Among tissue parasites the pigment was demonstrated by Stannard, McCoy & Latchford (1938) in *Trichinella* larvae and by v. Brand (1937) in a larval *Eustrongylides* from the mesentery of *Fundulus*.

The presence of haemoglobins in parasitic worms has been suggested as evidence that they were essential to supply oxygen to the oxidative systems (Davey 1938; v. Brand 1938). But not all authors presented evidence that the haemoglobins they observed were different from the haemoglobin of the host. Keilin (1925), however, observed that *Ascaris lumbricoides* contains two haemoglobins which could be distinguished spectroscopically from each other and from the host haemoglobin. These pigments were re-examined by Davenport (1949) and were found to have extremely high oxygen affinities, the consequence of a very low deoxygenation velocity. The oxygen equilibrium relations of the haemoglobins could not be determined directly but it was shown that *Ascaris* is capable of bringing about deoxygenation of the body-wall haemoglobin when kept under anaerobic conditions. Similarly Aducco (1889) observed that the haemoglobin of *Diectophyme* is extremely resistant to deoxygenation *in vacuo*.

Only one direct determination of the oxygen equilibrium curve of a haemoglobin from a parasitic nematode has been recorded. Wharton (1941) found that, at an unspecified temperature and pH, the haemoglobin of *Camallanus* was half saturated with oxygen at a tension of 8 mm. Under the same conditions a tension of 20 mm. was necessary for half saturation of the blood of the host, *Pseudemyss*. Under anaerobic conditions *Camallanus* could deoxygenate its haemoglobin. The oxygen affinity of this pigment is not therefore so high as that of the *Ascaris* haemoglobins described by Davenport, but is well adapted to take up oxygen at low tensions.

Because of its availability, large size, and powers of survival *in vitro*, *Ascaris lumbricoides* has been widely used as the type species in nematode physiology. Yet,

because of this large size *Ascaris* is aberrant and, since oxygen must enter by diffusion through the cuticle, unlikely to be fully aerobic under the most favourable conditions. More typical nematodes are smaller, and with a larger surface/volume ratio are better adapted to utilize the oxygen in the environment. Moreover, many of these smaller nematodes contain haemoglobin in great concentration and Wharton (1941) has shown that, in one of these, the haemoglobin does not share the unusual properties of the *Ascaris* haemoglobins. It was observed that two readily obtainable parasites of mammals, *Strongylus* spp. from the horse, and *Nippostrongylus muris* maintained artificially in the laboratory rat, contain such high haemoglobin concentrations and a study of these pigments was undertaken in order to compare their properties with those of *Ascaris* and *Camallanus*.

### 1. *NIPPOSTRONGYLUS MURIS*

*Nippostrongylus muris*, a trichostrongyle parasite of the brown and laboratory rats, is a small worm between 3 and 6 mm. long. The species was first described by Yokagawa (1921). In the description he refers to the red colour of the worm but did not identify the pigment. The parasite occurs in the small intestine of infected rats where the red masses of worms are clearly visible through the intestinal wall before this is opened.

When a few individuals are placed in saline on a slide, lightly compressed under a cover-slip, and observed with the microspectroscope as microscope ocular, the red colour of the worms is seen to have the spectrum of oxyhaemoglobin. The bands are nearer to the red than those of oxyhaemoglobin from the blood of the host. If the cover-slip is now ringed with vaseline to impede the ingress of air, and incubated at 37°C for about 30 min., the oxyhaemoglobin bands are replaced by the single band of haemoglobin. Reoxygenation rapidly occurs when air is again admitted. With larger numbers of worms under these conditions, deoxygenation of the haemoglobin is rapid and complete and is accompanied by a steady diminution in the activity of the worms until, when deoxygenation is complete, they are almost completely quiescent.

#### *Extraction of the haemoglobin*

Worms were removed from the opened intestine of the recently killed rat by scraping the mucosa with the back of a scalpel. *Nippostrongylus* lives with the anterior end deeply embedded in the crypts between the duodenal villi and cannot easily be removed without admixture with blood and tissue of the host. The worms were separated by repeated shaking in warm Ringer solution, allowing them to settle, and decanting off the saline with suspended debris. The resulting mass of active worms was filtered off on a small filter paper.

Attempts at extraction of the haemoglobin by grinding the worms in water or dilute buffer, even when this was accompanied by alternate freezing and thawing, gave extremely dilute solutions. After such treatment the debris still retained a bright red colour, indicating that the bulk of the haemoglobin is located in the

body wall of the parasite and not in the perienteric fluid. A more drastic method of extraction was therefore used.

Worms were dropped into liquid air in a small mortar and rapidly ground to a powder. The thawed powder was extracted with M/20 borate buffer pH 8.2 and centrifuged. The supernatant fluid was bright red and the worm debris at the bottom of the tube almost devoid of haemoglobin. From seven fairly heavily infected rats, about 6 ml. of a solution containing  $0.8 \times 10^{-4}$ M haematin could be obtained by this method.

*Absorption spectrum of the oxyhaemoglobin*

The positions of the absorption bands in the extracted oxyhaemoglobin, measured with the Hartridge spectrometer, were  $\alpha = 5777 \text{ \AA}$ ,  $\beta = 5405 \text{ \AA}$ . Identical readings were obtained when the observation was repeated on the pigment *in situ* in the worms. On the other hand the  $\alpha$ -band of rat blood oxyhaemoglobin is situated at  $5767 \text{ \AA}$ . *Nippostrongylus* must therefore synthesize haemoglobin or modify in some way that of the host. Figure 1 shows the spectrum of oxyhaemoglobin determined in the Hilger-Nutting spectrophotometer.

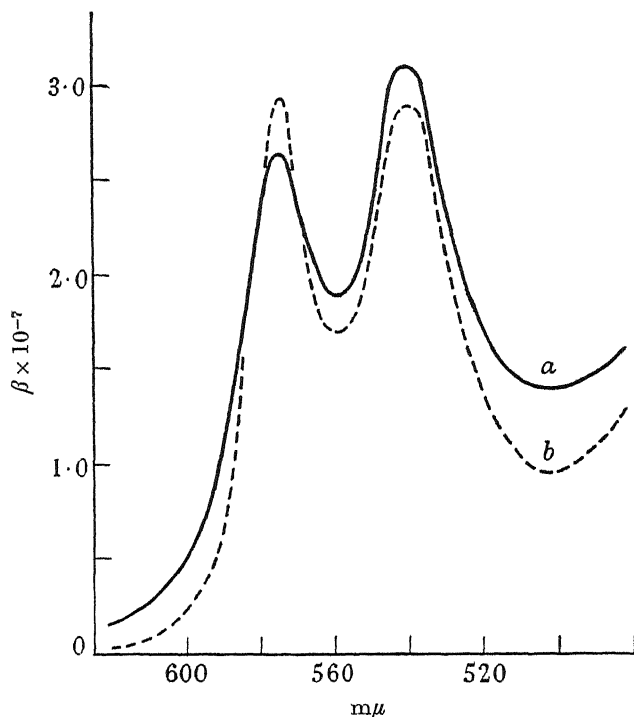


FIGURE 1. Absorption spectra of the oxyhaemoglobins of (a) *Strongylus* spp., (b) *Nippostrongylus muris*.

*The oxygen equilibrium curve*

Equilibration of the oxyhaemoglobin extract against a frequently renewed vacuum was found to bring about complete deoxygenation. The equilibration was carried out in a Thunberg tube. Deoxygenation proceeds relatively slowly, and

when a definite sequence of gentle shaking and re-evacuation was followed, such as would bring about deoxygenation of ox muscle haemoglobin in 5 min. the nematode pigment required about 30 min. When air was readmitted to the tube the haemoglobin became reoxygenated.

The oxygen equilibrium curve was determined by the micromethod of Hill (1936) with slight modification to allow the use of the very small volumes available. In this method a known volume of haemoglobin solution of known concentration is deoxygenated in a Thunberg tube. Amounts of oxygen, which can be calculated, are then added as oxyhaemoglobin solution saturated with air, through the side arm of the tube. After equilibration the effect of the added oxygen upon the degree of oxygenation of the haemoglobin in the tube is measured by matching its spectrum against that of a variable optical mixture of haemoglobin and oxyhaemoglobin, in the cups of the spectrophotometer described by Hill (1936).

### Method

The haemoglobin concentration was estimated by converting to globin haemochromogen and measuring the light absorption in the  $\alpha$ -band of this compound in the Hilger-Nutting spectrophotometer. The absorption constant  $\beta$  is given by the formula:

$$\beta = \frac{1}{cd} \log_e \frac{I_0}{I},$$

where:  $c$  is the haematin concentration in g.mol./ml.

$d$  is the thickness of the absorbing layer.

$I_0$  and  $I$  are the intensities of incident and transmitted light respectively.

Starting from pure haemin and pig globin  $\beta 558.5 = 0.64 \times 10^8$ . The details of this method have been described by Davenport (1949).

In each vacuum tube 2 ml. of a solution of the oxyhaemoglobin containing, usually,  $0.6 \times 10^{-4}$  M haematin, was used. During deoxygenation, evaporation from this small volume caused a serious change in concentration. To counteract this the tubes were calibrated to contain 2 ml. and an additional 0.3 ml. of water added before evacuation. Evaporation was allowed to go on until the level of the solution fell to the graduation mark.

When deoxygenation was complete successive small amounts of *Nippostrongylus* oxyhaemoglobin solution were admitted through the side arm from a 2 ml. graduated pipette attached by a short length of rubber tubing. After each addition equilibration was continued for 10 min., until the spectrum of the solution in the tube showed no further change. The absence of further change showed that, under the conditions of the experiment, the nematode extracts had no appreciable oxygen uptake.

It was necessary to use human oxyhaemoglobin and haemoglobin as the standards against which matching was carried out, in the spectrophotometer cups. Apart from the difference in position of the absorption bands, the spectra of the two oxyhaemoglobins are otherwise similar and no difficulty was experienced in making the comparison.



In the course of these experiments, considerable difficulty was experienced in avoiding a partial denaturation of the haemoglobins or their partial oxidation to methaemoglobin. Exposure to low tensions of oxygen during the somewhat prolonged deoxygenation favoured oxidation, and attempts to speed deoxygenation by more vigorous shaking, or by warming the tube, hastened denaturation. It was therefore particularly important, when air was readmitted to the tube at the end of an experiment, to make certain that the bands of oxyhaemoglobin were restored at their original intensity. More than half the experiments which were carried out failed to satisfy this criterion of the absence of secondary change.

The pressure of oxygen in the tube in mm. Hg corresponding to the percentage of oxyhaemoglobin determined as described above was calculated from the formula given by Hill (1936),

$$\text{O}_2 \text{ pressure in mm. Hg} = P \times \frac{v_1[\text{O}_2] - (v_1 + v_2)[\text{HbO}_2]}{(v_1 + v_2) + A(v_0 - v_1 - v_2)},$$

where  $[\text{O}_2]$  = total concentration of  $\text{O}_2$  in the fluid added, i.e. dissolved  $\text{O}_2$  + that present as oxyhaemoglobin.

$[\text{HbO}_2]$  = concentration of haemoglobin.

$v_0$  = total internal volume of the tube (ml.)

$v_1$  = volume of fluid added from the pipette (ml.)

$v_2$  = initial volume of fluid in the tube (2 ml.)

$A$  = quotient of the concentration of  $\text{O}_2$  in gas phase divided by the concentration of  $\text{O}_2$  dissolved in the liquid independently of the pressure.

$P$  = the factor for converting concentration of dissolved  $\text{O}_2$  into the equivalent pressure in mm. Hg.

( $A$  and  $P$  can be calculated from solubility data.)

The experiments were carried out at pH 8.2 (M/20 borate) and at temperatures between 17.5 and 19°C.

For comparison of the results with *Nippostrongylus* haemoglobin the dissociation curve of ox muscle haemoglobin was determined under the same conditions. The muscle haemoglobin was prepared by the method described by Hill (1939) from shin beef and dialyzed to equilibrium with M/20 borate buffer pH 8.2. In these experiments muscle haemoglobin and oxyhaemoglobin were used as standards in the spectrophotometer cups.

### Results

The result of a typical experiment is given in table 1. Estimation of the percentage oxyhaemoglobin with the spectrophotometer is most accurate in the range 25 to 75 %, when the readings do not deviate by more than 3 %. The amount of oxygenated haemoglobin which must be added to 2 ml. of the *Nippostrongylus* haemoglobin in the vacuum tube, in order to cover this range, is very small. The error involved in calculating the oxygen tension within the tube is therefore greater than with a haemoglobin having a lower affinity for oxygen.

Figure 2 summarizes the results of these experiments. With both haemoglobins, when the logarithm of the calculated oxygen tension is plotted against the logarithm

if the ratio of oxyhaemoglobin to haemoglobin at that tension, the points fall, within the limits of the experimental error, upon a straight line of slope  $45^\circ$ , corresponding to a rectangular hyperbola. The corresponding oxygen equilibrium

TABLE 1. OXYGEN EQUILIBRIUM CURVE OF *NIPPOSTRONGYLUS* HAEMOGLOBIN

Volume of vacuum tube ( $v_0$ ) = 18.85 ml. Hb concentration equivalent to  $0.6 \cdot 10^{-4}M$  haematin.  
Initial volume HbO<sub>2</sub> solution = 2 ml., pH 9.2, 19°C

added from pipette (ml.)	mean color- meter scale reading (cm.)	percentage HbO <sub>2</sub>	O <sub>2</sub> (mm.)
0.308	0.62	31.0	0.064
0.514	1.13	56.5	0.095
0.803	1.35	67.5	0.171
1.45	1.60	80.0	0.366

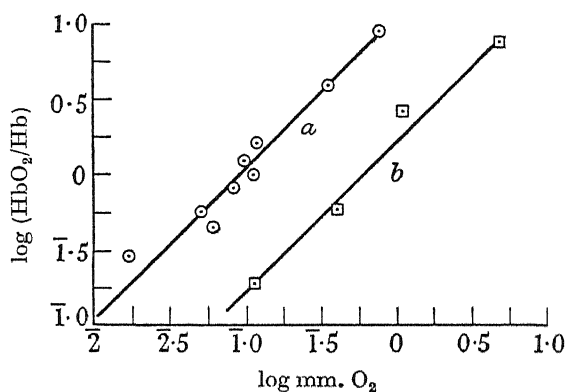


FIGURE 2. Oxygen equilibrium of (a) *Nippostrongylus* haemoglobin, and (b) ox muscle haemoglobin.  $\log \text{HbO}_2/\text{Hb}$  plotted against  $\log$  pressure O<sub>2</sub> in mm. Hg.

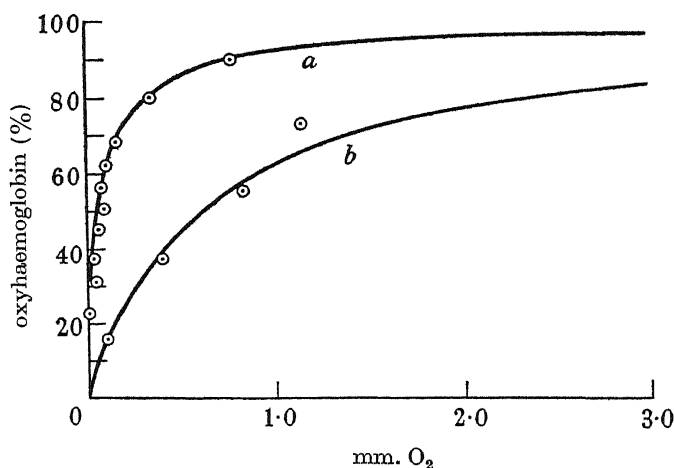


FIGURE 3. Oxygen equilibrium curves of solutions of (a) *Nippostrongylus* haemoglobin, and (b) ox muscle haemoglobin. Hb equivalent to  $0.6 \times 10^{-4}M$  haematin, pH 9.2, 19°C.

curves are shown in figure 3. Ox muscle haemoglobin at 18°C, pH 9, was half saturated at an oxygen tension of 6 mm. Hg. Under the same conditions a tension of about 0.1 mm. is required for half saturation of the *Nippostrongylus* haemoglobin.

## 2. *STRONGYLUS* SPP.

The dull red colour of the species of *Strongylus* which inhabit the large intestine of the horse, has led to their being known collectively as 'red worms'. Superficial observation of the worms *in situ* gives the impression that they are engorged with the blood of the host. Host haemoglobin is undoubtedly ingested, but Rogers (1940) has shown that digestion is so rapid that the intestinal contents of the parasite are coloured dark brown by masses of haematin and contain very little unchanged haemoglobin.

The red colour was found, in the present work, to be due to a bright red pigment in the perienteric cavity of the parasite, visible through the thin body wall. As soon as possible after slaughtering, female *Strongylus* were removed from the gut of the host and washed for one hour in Ringer solution at 37°C. Perienteric fluid was removed from the worms by immersing the anterior end in a watch glass containing M/20 phosphate buffer pH 7, puncturing the body wall in the region of the muscular pharynx, and gently squeezing the posterior end of the parasite. The gut of the worm ruptures very easily if removal of the whole of the perienteric fluid is attempted.

The amount of pigment in the perienteric fluid shows great individual variation. Occasional specimens yielded a fluid having only a pale pink colour but an average batch of 30 worms gave about 5 ml. of a diluted fluid containing  $3.0 \times 10^{-4}$  M haematin.

### *Spectroscopic properties*

The spectrum of the suitably diluted perienteric fluid, viewed with the micro-spectroscope, exhibits two absorption bands at 5781 and 5400 Å. Compared with the spectrum of *Nippostrongylus* oxyhaemoglobin the bands are more diffuse and closely resemble the spectrum of *Ascaris lumbricoides* oxyhaemoglobin (Davenport 1949). Figure 1 shows this spectrum. When  $K_3Fe(CN)_6$  is added to the solution the typical spectrum of acid methaemoglobin slowly appears with maxima at 637 and 500 mμ. This, upon reduction with  $Na_2S_2O_4$  is immediately replaced by the single band of haemoglobin at 555 mμ and, when the solution is aerated, the two bands originally observed reappear. The relation of the pigment in the perienteric fluid to these derivatives is thus that of oxyhaemoglobin.

### *Deoxygenation of the oxyhaemoglobin*

A Thunberg tube containing 3 ml. of the diluted perienteric fluid in M/20 phosphate, pH 7 was evacuated. Equilibration against the vacuum by gentle shaking alternating with frequent re-evacuation failed, in 3 hr., to bring about a change in the spectrum of the fluid in the tube.

The addition of  $Na_2S_2O_4$  to the oxyhaemoglobin caused a slow replacement of the two absorption bands by the single band of haemoglobin. At room temperature

the slow change may easily be followed and closely resembles the similar slow reaction which has been reported for the oxyhaemoglobin in the perienteric fluid of *Ascaris*. The supply of the *Strongylus* pigment was too small to permit a full investigation of the reaction but measurements of its velocity were made at pH 7 and 11.5°C.

The procedure was precisely as was described for the measurement of deoxygenation velocity of *Ascaris* oxyhaemoglobins. Into a Thunberg tube 3 ml. of the oxyhaemoglobin solution containing  $0.7 \times 10^{-4}$  M haematin, was pipetted. The hollow stopper containing 10 mg.  $\text{Na}_2\text{S}_2\text{O}_4$  was replaced and the tube evacuated. The reaction was started by tipping the hyposulphite into the body of the tube and shaking to ensure solution, and its progress measured by comparing the spectrum of the reacting solution with that of a variable optical mixture of haemoglobin and oxyhaemoglobin in the cups of the spectrophotometer. Since the available amounts of *Strongylus* haemoglobin were so small, human haemoglobin and oxyhaemoglobin were used for the comparison. Complete matching of the whole spectrum was impossible but it was found that if the effective concentration of the human oxyhaemoglobin was adjusted to obtain matching of the intensities of the  $\beta$ -bands at the beginning of the reaction, and the haemoglobin in the other cup was of the same concentration, a fairly satisfactory estimate of the progress of the reaction could be obtained by comparing the  $\beta$ -bands of the two haemoglobins.

The time for half completion of the reaction under these conditions was found to be  $750 \pm 80$  sec. When the logarithms of the percentage oxyhaemoglobin are plotted against time a straight line is obtained within the rather large experimental error imposed by the use of a different haemoglobin as comparison standard. Under the same conditions of pH and temperature  $t_{50}$  for the deoxygenation of *Ascaris* perienteric fluid haemoglobin was  $600 \pm 50$  sec.

## DISCUSSION

The most striking property of the two nematode haemoglobins is their great affinity for oxygen. In dilute solution at 19°C, *Nippostrongylus* haemoglobin is half saturated with oxygen at a pressure of less than 0.1 mm. Hg. Of other haemoglobins for which the equilibrium curve has been determined, only one possesses a comparably high oxygen affinity. Keilin & Wang (1945) extracted from the root nodules of leguminous plants a haemoglobin which, at 15°C, is also half saturated at below 0.1 mm. oxygen pressure. In spite of this high oxygen affinity rapid and complete deoxygenation of the haemoglobin occurs when *Nippostrongylus* is incubated under conditions of oxygen deficiency. Thus, whilst the haemoglobin is adapted to take up oxygen at extremely low tensions of the gas, the oxidative enzyme systems of the parasite are able to utilize this bound oxygen.

Since nematodes are without specialized respiratory organs or a blood vascular system, oxygen must enter by diffusion through the general body surface or, in those forms which suck the arterial blood of the host, through the gut wall. In an oxygen deficient environment, small size, by increasing the surface/volume ratio, is an important factor in facilitating the utilization of the available oxygen.

Not only is *Nippostrongylus* a small worm but normally it lives attached to, or embedded between, the richly vascular folds of the host intestinal mucosa, which receives blood from the oxygen-rich portal circulation. Although the primary function of these folds is to increase the area available for the absorption of the products of digestion, their presence must also increase the amount of oxygen diffusing into the intestinal contents. Slater (1925) has pointed out that oxygen analyses of the strongly reducing contents of the intestine give no indication of the oxygen supply available to intestinal parasites unless allowance is made for the steady supply available by diffusion from the host intestinal wall during life.

The haemoglobin of *Nippostrongylus* occurs principally in the body wall of the worm and is invariably present in high concentration. It is therefore well located to take up oxygen diffusing through the cuticle of the nematode. Deoxygenation of the haemoglobin under conditions of oxygen deficiency is accompanied by an increasing degree of inactivity. Under similar conditions Davey (1938) observed inactivity of sheep trichostrongyles but he did not correlate this with deoxygenation of the haemoglobin he observed in those worms. On the other hand the quiescence of *Ascaris* in the absence of oxygen which led Slater (1925) to question the total anaerobiosis of this parasite increases concurrently with deoxygenation of the body-wall haemoglobin (Davenport 1949). In *Nippostrongylus* recovery of active movement when air is readmitted, with reoxygenation of the haemoglobin, is rapid and spectacular and the cycle of events may be repeated several times without apparent injury to the worms. Referring to *Ascaris*, Slater (1925) concluded that '... although the worms are capable of prolonged existence in the absence of air, they achieve this only by cutting down their movements to a minimum, and that for their normal metabolism they require a supply of oxygen'. This conclusion would appear to apply to *Nippostrongylus* also and the haemoglobin in the worm serves as a short period store of oxygen available for rapid transference to the oxidative enzyme system during periods of temporary oxygen deficiency.

There is no evidence that the haemoglobin of *Strongylus* possesses a respiratory function. The high oxygen affinity is here the consequence of an extremely low deoxygenation velocity, a property it shares with the haemoglobin in the perienteric fluid of *Ascaris* (Davenport 1949). In the absence of oxygen *Strongylus* becomes moribund without visible change in the spectrum of the haemoglobin, an indication that the oxidative enzymes of the worm are unable to utilize this oxygen. Laser (1944), working with *Ascaris*, has suggested that since methaemoglobin is a weak peroxidase, the function of haemoglobin in this worm may be the catalytic elimination of  $\text{H}_2\text{O}_2$  produced during oxidative metabolism, but he gave no direct evidence that this was their role.

In *Strongylus* and *Ascaris*, and in contrast to *Nippostrongylus*, the haemoglobin content is highly variable. *Ascaris* is not known normally to suck the blood of the host but in occasional individuals a high haemoglobin content is associated with the presence of high haematin concentrations in the gut (Davenport 1949). On the other hand *Strongylus* usually has the gut distended with material coloured black by haematin derived from the blood of the host (Rogers 1940). The haemoglobin concentration in the perienteric fluid, immediately adjacent to the gut, is normally

many times greater than is ever observed in *Ascaris*. The picture is in some ways analogous to that of the pigments investigated by Wigglesworth (1943) in *Rhodnius*, a blood-sucking bug. Although most of the haemoglobin ingested by the insect is broken down to haematin in the gut, a small amount is absorbed and circulates in the blood as parahaematin. The concentration of circulating parahaematin in *Rhodnius* is subject to great variation and can be increased by injecting haemoglobin into the haemocoel. Wigglesworth could attribute no function to the pigment. It is possible therefore that the aberrant haemoglobins in the perienteric fluid of *Ascaris* and *Strongylus* are functionless by-products of the nutrition of the worms differing from the parahaematin of *Rhodnius* in that they retain, in a much modified form, the essential properties of haemoglobin.

The contrast between the properties of *Strongylus* and *Nippostrongylus* haemoglobins emphasize the danger in the assumption, referred to in the introduction to this paper, that the presence of haemoglobin in parasitic nematodes is evidence that they fulfil a respiratory role.

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# *Ascaris* haemoglobin as an indicator of the oxygen produced by isolated chloroplasts

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## INTRODUCTION

A technique for using haemoglobin as an indicator of oxygen produced by the photosynthetic activity of green leaves was first described by Hoppe-Seyler (1879). The method has been used with notable success by Hill (1937, 1939) and by Hill & Scarisbrick (1940) in their observations on the sub-cellular activity of isolated chloroplasts. The quantities of oxygen evolved in their experiments were small, and since the sensitivity of measurement was dependent upon the affinity of the haemoglobin for oxygen, it was desirable to select a haemoglobin having as high an oxygen affinity as possible. Hill (1936), however, had shown that muscle haemoglobin in dilute solution has a greater affinity than the blood haemoglobin of the same mammal. Thus at pH 8 and 19°C he found ox muscle haemoglobin to be half saturated at an O<sub>2</sub> pressure of 0.7 mm. compared with 1.8 mm. for ox blood haemoglobin under the same conditions. Using muscle haemoglobin as indicator, Hill (1939) was able to show that illuminated chloroplasts in presence of leaf extracts evolve oxygen to a pressure of 1 mm. Hg and in presence of ferric oxalate to a pressure of 4 mm. Hg.

Haemoglobins are now known which have oxygen affinities higher than that of mammalian muscle haemoglobin. In particular it has been shown that *Ascaris lumbricoides*, a nematode parasitic in the pig, contains two haemoglobins remarkable for their extreme resistance to deoxygenation when they are equilibrated *in vacuo* (Davenport 1949). Because of this property the standard methods for the determination of the oxygen equilibrium curves could not be used. Hill (1939) had used a haemoglobin of known oxygen affinity to measure the tensions of oxygen produced by illuminated chloroplast systems. The object of the present paper is to compare the response of ox muscle haemoglobin and the *Ascaris* haemoglobins to oxygen produced photochemically under standard conditions and thus to obtain an indication of the relative affinities of the haemoglobins.

## PRELIMINARY OBSERVATIONS

In the basic procedure of Hill (1939), suspensions of chloroplasts are introduced into an evacuated Thunberg tube containing haemoglobin and either an aqueous extract of leaf acetone powder or a solution of ferric oxalate. The tube is then illuminated and oxygen estimated spectroscopically as oxyhaemoglobin.

The *Ascaris* haemoglobins are not appreciably deoxygenated when they are equilibrated against a vacuum for short periods. It was shown previously (Davenport 1949) that their high oxygen affinity is the result, principally, of an extremely low deoxygenation velocity. Even in the presence of powerful reducing agents such as sodium hyposulphite the reaction proceeds slowly enough for measurement by static methods. These haemoglobins could not therefore be used in the detection of oxygen evolved in Hill's reaction until a method had been worked out for their deoxygenation in the absence of powerful reducing agents.

It was shown by Hill (1939) that illuminated chloroplast suspensions alone do not evolve oxygen but when an aqueous extract of acetone powder of leaves is added to the system oxygen is immediately evolved. These leaf extracts also contain reducing systems capable of bringing about a slow deoxygenation of the *Ascaris* haemoglobins. 2 g. of an acetone powder of young leaves of *Lamium album*, prepared according to the directions of Hill (1939), were stirred into 20 ml. of water and, rapidly squeezing through muslin, the extract was centrifuged. To 2.5 ml. of a solution of *Ascaris* perienteric fluid haemoglobin, pH 7.4 and containing  $10^{-4}$  M haematin, in a Thunberg tube, 1.5 ml. of the extract were added and the tube evacuated. After 18 hr. incubation at room temperature, between 80 and 90 % of the haemoglobin was in the deoxygenated form. Through the side arm of the evacuated tube 0.4 ml. of a suspension of *Stellaria media* chloroplasts, prepared by the standard method of Hill & Scarisbrick (1940) was added. Upon exposing the tube to the light of a projection lamp the absorption spectrum of oxyhaemoglobin appeared and grew in intensity until it corresponded to 100 % oxyhaemoglobin. The long period of incubation does not therefore destroy the capacity of the leaf extracts to promote an evolution of oxygen when chloroplasts are subsequently added and illuminated. Moreover, the haemoglobin undergoes no detectable secondary change.

Control experiments, similar to those of Hill (1939), showed that (a) illumination of the tube before the addition of chloroplasts had no effect, (b) chloroplast suspensions which had been heated to 100°C or stored for 18 hr. at 4°C were inactive.

Because of the necessity for a preliminary incubation of haemoglobin and leaf extract it was not possible to determine whether chloroplasts alone were able to bring about reoxygenation of the *Ascaris* haemoglobin. Nevertheless the continued activity of the system after this incubation made possible a direct comparison of the response of *Ascaris* haemoglobin and mammalian haemoglobin to oxygen evolved in the reaction.

#### MATERIALS AND METHODS

As far as possible the arrangement of the apparatus, the method of preparation of ox muscle haemoglobin, suspensions of chloroplasts of *Stellaria media*, and the measurement of the pigment concentration in these preparations followed the information given by Hill (1939) and Hill & Scarisbrick (1940). The extraction of haemoglobin from the perienteric fluid and from the body wall of *Ascaris* has been described previously (Davenport 1949). Their concentrations were measured as cyanmethaemoglobin; the absorption constant at  $544 \mu$  being taken as  $2.57 \times 10^7$ .



All experiments were carried out in M/30 phosphate buffer pH 7.4. Hill found chloroplasts to have maximum photochemical activity at pH 8.0, but the lower pH was chosen because of the increased stability of the *Ascaris* haemoglobins in less alkaline solution. On the day before an experiment tubes containing the appropriate haemoglobin and leaf extract were set up and evacuated as described in the previous section and incubated on the bench overnight. In order to obtain standard conditions muscle haemoglobin, when this was used, was also incubated in the presence of the leaf extract. After 18 hr. incubation, deoxygenation of the *Ascaris* haemoglobin was usually sufficiently advanced for the addition of chloroplast suspensions. Special precautions were necessary during this addition to avoid the accidental introduction of oxygen, since any oxyhaemoglobin so produced could not be deoxygenated by re-evacuation.

The leaves of *Stellaria media*, after grinding in M/30 phosphate buffer pH 7.4, containing 13 % sucrose, were filtered into a Thunberg tube. The tube was evacuated and gently shaken until frothing ceased. The vacuum was then released and part of the suspension taken up in a drawn-out pipette, dipping well below the surface, and introduced into the side arm of the reaction tube. Bubbles of air, entangled in the tap grease at the base of the arm, were dislodged with the point of the pipette. The side arm was filled completely and several drops of suspension allowed to overflow, in order to displace the first introduced portions which had been in contact with air. A 1.0 ml. graduated pipette, containing the rest of the suspension, was then attached to the side arm by a short length of rubber tubing and the required volume of suspension cautiously admitted to the reaction tube. For each tube a fresh chloroplast suspension was prepared. The reaction was begun by exposing the tube to the light of the projection lamp exactly 15 min. after grinding of the *Stellaria* leaves commenced.

A 500 W projection lamp was the source of photochemical energy and this was arranged in relation to the spectrocoulometer precisely as described by Hill & Scarisbrick (1940). Light intensity was varied by sliding resistance, and illumination at the reaction point was measured with a selenium cell lux-meter. This method of measurement has the advantage that the spectral response curve of this type of cell includes the wave-lengths above  $600\ \mu$  which are most active in promoting the photochemical reaction involving chlorophyll. Most of the experiments were carried out at 7300 lux.

After addition of the leaf extract the haemoglobin concentration in the tubes was equivalent to  $0.6 \times 10^{-4}$  M haematin. The tubes had an effective thickness of 1.5 cm. The production of oxyhaemoglobin was followed by comparing the spectrum of the reacting system with that of an optical mixture of the same haemoglobin and oxyhaemoglobin in the cups of the spectrocoulometer.

## RESULTS

No difference could be detected in the response of the two *Ascaris* haemoglobins to oxygen evolved in the chloroplast reaction. Figure 1*a* shows the result of an experiment with perienteric fluid haemoglobin. Oxygenation of the haemoglobin

proceeded at a steady rate until full saturation was attained, in about 270 sec. If it is assumed that the oxygen output of the system over this period was constant then the oxygen affinity of the haemoglobin is so high that, within the limits of a 5 % error in the method of measurement, the percentage oxyhaemoglobin was proportional to the oxygen concentration up to complete saturation. This result may be contrasted with the data given by Hill (1936) for ox muscle haemoglobin which has a relatively high oxygen affinity. At pH 7 and 19°C the pigment is 58 % oxygenated at 1 mm. oxygen pressure whereas at 2 mm. pressure only a further 17 % of the haemoglobin becomes oxygenated.

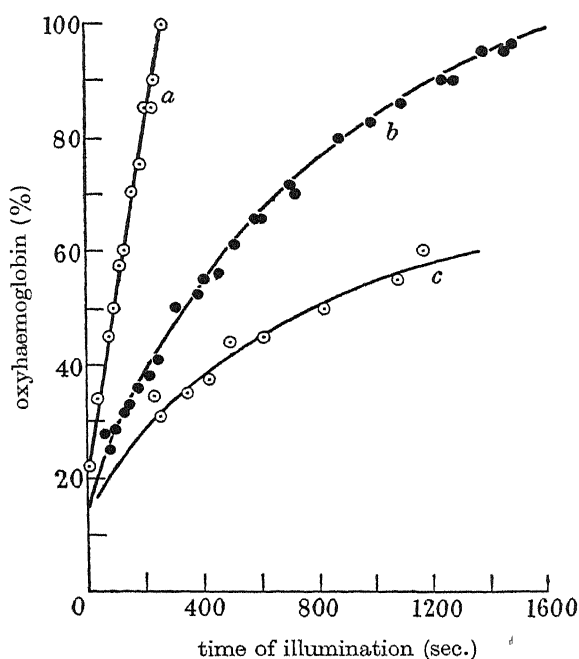


FIGURE 1. Experimental curves showing oxygenation of *Ascaris* perienteric fluid haemoglobin in presence of *Stellaria media* chloroplasts and extract of *Lamium album* leaves. Haemoglobin =  $0.6 \times 10^{-4}$  M haematin. 23°C, pH 7.4, 7300 lux. Curve a, 0.4 ml. chloroplast suspension; leaf extract prepared from acetone powder No. 1. Curve b, 0.4 ml. chloroplast suspension. Acetone powder No. 3. Curve c, 0.2 ml. chloroplast suspension. Acetone powder No. 3.

In the series of experiments of which figure 1a is a typical result no direct comparison with ox muscle haemoglobin was made. In subsequent experiments where this was done the reaction proceeded more slowly. The difference was found to be due to variations in the activity of the leaf extracts. Extracts from different acetone powders of *Lamium album* leaves showed wide variations in their activity but successive extracts from a single preparation over a period of one week showed no appreciable differences in their activity. Figure 1b is the result of a typical experiment in a series where a less active acetone powder was used. The initial rate of oxygenation, about half that in figure 1a, was not maintained but diminished

slowly with time until complete saturation was reached in 1600 sec. By varying separately the conditions of this experiment it was possible to examine the factors associated with this diminution.

(a) *Chloroplast concentration*

Figure 1c is the result of an experiment carried out under the same conditions as figure 1b except that the chlorophyll concentration was reduced to half. The initial oxygenation rate is also about half that at the higher chlorophyll concentration and complete saturation of the haemoglobin was not reached. By drawing tangents to these curves the oxygenation rates at convenient time intervals may be determined. The values, expressed as percentage oxyhaemoglobin/sec., form two series which diminish exponentially with time of illumination as shown graphically in figure 2. Extrapolation of the points to zero time gives the true initial rate of oxygenation. With  $0.4 \times 10^{-4}$  M chlorophyll this was 0.13 % oxyhaemoglobin/sec. compared with 0.07 % at half that chlorophyll concentration. This result agrees with the findings of Hill (1939).

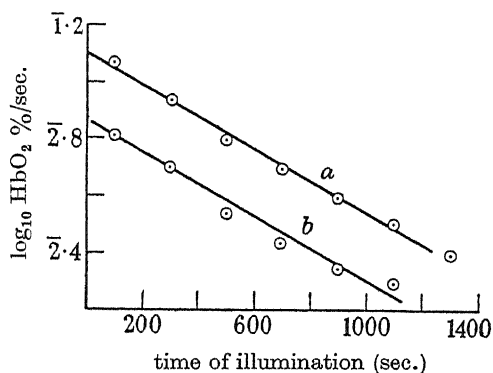


FIGURE 2. Oxygenation of *Ascaris* perienteric fluid haemoglobin by illuminated chloroplasts. Diagram to show diminution in rate of oxygenation in figures 1b and 1c. a, data from figure 1b. b, data from figure 1c.

More important, from the point of view of the properties of the *Ascaris* haemoglobins, is the slope of the points in figure 2. This is a measure of the rate at which the oxygenation velocity decreases with time and is the same at both chlorophyll concentrations in spite of the difference in the initial oxygenation velocity. At the higher chlorophyll concentration oxygenation of the haemoglobin proceeded to completion and covers the range 15 to 95 % saturation but at the lower concentration only 60 % saturation was reached. The rate of decrease of the oxygenation velocity is thus independent of the level of saturation of the haemoglobin and the decrease is not therefore an effect due to the oxygen equilibrium relations of the pigment. The results suggest that during illumination the oxygen output of the photochemical reaction declines exponentially with time.

In the experiment shown in figure 1b, the rate of oxygenation falls to half the initial rate in 500 sec. With a more active photochemical system, such as gave the

result in figure 1*a*, the haemoglobin becomes completely oxygenated too rapidly for the decline in oxygen production with time to come within the experimental error of the method of measurement.

(b) *Light intensity*

At the point of exposure of the reaction tubes the maximum illuminating power of the projection lamp was found to be 28,000 lux. Comparisons were made between the effect of this increased illumination and the 7300 lux used in the other experiments.

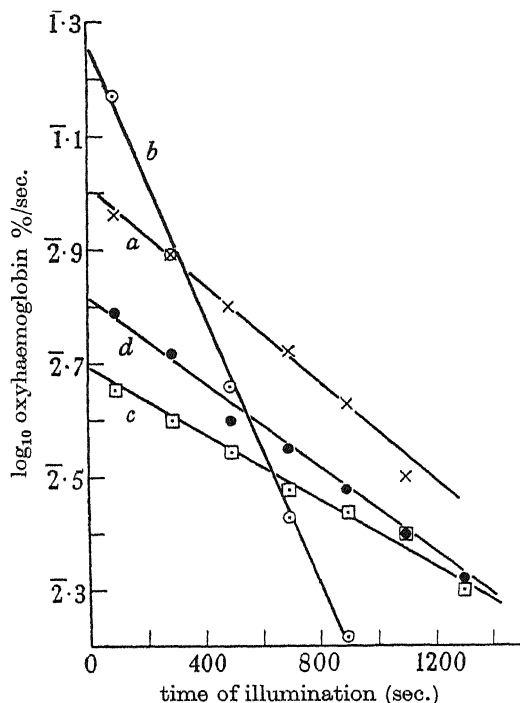


FIGURE 3. Diagram to show influence of some factors on the rate of oxygenation of *Ascaris* perienteric fluid haemoglobin during illumination in the chloroplast reaction. Haemoglobin =  $0.6 \times 10^{-4}$  M haematin. 0.4 ml. chloroplast suspension, 23°C, pH 7.4. *a*, 7300 lux, haemoglobin and leaf extract *in vacuo* 18 hr., fresh chloroplasts. *b*, as *a* but 28,000 lux. *c*, 7300 lux, haemoglobin and leaf extract *in vacuo* 18 hr., chloroplasts then added and stored in dark, 140 min. before illumination. *d*, as *a* but haemoglobin and leaf extract *in vacuo* 42 hr.

The comparison is illustrated by the results in figure 3*a* and *b*, plotted in the manner described above for figure 2. The initial rate of oxygenation at 28,000 lux is 0.186 %/sec. compared with 0.10 %/sec. at 7300 lux, but the rate diminishes more rapidly at the higher illumination. Thus a reduction to half the initial rate occurs in 250 sec. at 28,000 lux and in 650 sec. at 7300 lux. It is inferred that although increased light intensity raises the initial oxygen output of the photochemical system, it also accelerates the exponential decline in oxygen output which accompanies illumination.

(c) *Ageing of chloroplasts*

Hill (1939) found that the activity of chloroplasts stored in buffered sucrose solution diminishes rapidly. In general he found a 50 % loss of activity in 2 hr. at room temperature. In view of the long duration of some experiments with *Ascaris* haemoglobin it was considered to be of greater importance to measure the rate of this inactivation when the chloroplasts were stored in presence of the plant extract and haemoglobin *in vacuo*.

When a comparison was made between different chloroplast suspensions of similar initial activity the results showed considerable variation, but half the activity, measured by the initial rate of oxygenation of the haemoglobin, was lost in from 1½ to 2½ hr. at room temperature.

An experiment of this kind is given as figure 3c and was carried out under the same conditions as figure 3a except that after introduction of the chloroplasts the tube was stored in the dark for 140 min. In this time the initial oxygenation rate fell from 0.10 to 0.048 % oxyhaemoglobin/sec., a 50 % loss in activity. Comparison of the slope of the curves *a* and *c* in figure 3 indicates that, in spite of their lower initial activity, the oxygen production of the stored chloroplasts diminishes during illumination slightly less rapidly than when fresh chloroplasts are used and the initial activity falls to half in about 1100 sec.

(d) *Storage of the haemoglobin with leaf extract*

The necessity for a preliminary incubation of the *Ascaris* haemoglobins with leaf extract in order to bring about deoxygenation prevented an investigation into the activity of the fresh leaf extracts, in presence of these haemoglobins. Results with ox muscle haemoglobin will be given below but a number of experiments were carried out with *Ascaris* haemoglobin after longer incubation periods than 18 hr. In spite of some variation in the results a further incubation period of 24 hr., 42 hr. in all, results in a 50 % loss of initial oxyhaemoglobin formation when chloroplasts are added and the tubes illuminated. Figure 3d illustrates a typical experiment and shows also that the diminution of the rate of oxygen production during illumination remains unaffected in spite of the lower initial rate.

MAMMALIAN BLOOD AND MUSCLE HAEMOGLOBIN

From the above results it has been possible to show, without direct comparison with haemoglobins of known oxygen affinity, that, within a 5 % error in the method of measurement, oxygenation of the *Ascaris* haemoglobins proceeds to saturation in direct proportion to the oxygen concentration. Control experiments with human blood haemoglobin and ox muscle haemoglobin served further to confirm this extreme oxygen affinity.

The results with human blood haemoglobin were uniformly negative. Greatly increased light intensity, concentration of the plant extracts and of the chloroplast suspensions failed to bring about any perceptible formation of oxyhaemoglobin. When ox muscle haemoglobin was substituted there was, however, a small but measurable response.

The experiment shown in figure 4, inset *a*, was carried out by admitting chloroplasts to the tube containing leaf extract and muscle haemoglobin without a preliminary incubation period. The initial rate of oxygenation is rapid but decreases until, at 400 sec., a maximum of 30 % oxyhaemoglobin is present. With continued illumination a slow deoxygenation occurs. Where the muscle haemoglobin was first incubated for 18 hr. *in vacuo* with the leaf extract the response was similar but both the initial rate of oxygenation and the level of oxygenation at which equilibrium occurred, were less. In general this preliminary incubation resulted in a 50 % decrease in these values. Thus, in the direct comparison with an *Ascaris* haemoglobin which ultimately became fully oxygenated, only 15 % muscle oxyhaemoglobin was formed. This corresponds to an oxygen tension of less than 0.1 mm. Hg. The contrast between the responses of the two haemoglobins is well shown in figure 4*A* and *B*. An important feature illustrated by this figure is that, although the initial rates of oxygenation of the two pigments are similar, muscle haemoglobin attains maximum oxygenation in 400 sec., that is 1200 sec. before the *Ascaris* haemoglobin reaches full saturation.

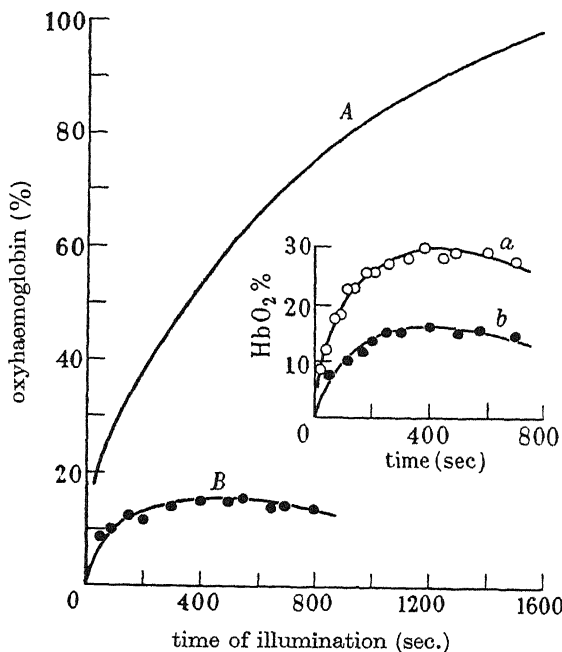


FIGURE 4. Comparison of the oxygenation of *Ascaris* perienteric fluid haemoglobin and ox muscle haemoglobin by oxygen produced in the chloroplast reaction under the same conditions. Haemoglobin =  $0.7 \times 10^{-4}$  M haematin. 23° C, pH 7.4, 0.4 ml. chloroplast suspension, 7300 lux. Curve *A*, *Ascaris* perienteric fluid haemoglobin. Curve *B*, ox muscle haemoglobin. Inset, muscle haemoglobin *a* reaction immediately after addition of leaf extract and evacuation of tube, *b* haemoglobin and leaf extract *in vacuo* 18 hr.

#### DISCUSSION

The essential property of haemoglobin is its capacity to enter into a labile combination with molecular oxygen without undergoing oxidation. When the change in spectrum which accompanies oxygenation is used in the measurement of

oxygen in solution the oxygen equilibrium curve of the haemoglobin may be regarded as a calibration curve of the method. If small amounts of oxygen are added to deoxygenated haemoglobin the increase in the level of oxygenation provides a measure of the oxygen tension in the solution and if the concentration of haemoglobin is known the quantity of oxygen can be calculated. This provides the basis for the method introduced by Hill (1937) for the measurement of oxygen evolved when isolated chloroplasts are illuminated. But in the chloroplast reaction the position is complicated by the presence of reducing systems capable of competing with the haemoglobin for oxygen evolved in the reaction. Hill (1939) observed that muscle haemoglobin, oxygenated in the presence of isolated chloroplasts and the necessary reagents in the light, undergoes a rapid deoxygenation in darkness. During illumination also, the maximum level of oxygenation attained corresponds to the oxygen tension at which the opposing oxygen production and oxygen consumption are in equilibrium. Using leaf extracts to promote the chloroplast reaction with muscle haemoglobin, Hill (1939) was able to obtain levels of oxygenation corresponding to an oxygen tension of 1 mm. Hg. This value, however, gives no information of the rate of oxygen production at equilibrium.

Muscle haemoglobin at 23°C is half saturated with oxygen at a tension of 0.6 mm. Hg and the form of the oxygen equilibrium curve is a rectangular hyperbola (Hill 1936). During the initial stages of oxygenation, therefore, the tension of oxygen in solution is for practical purposes negligible and the influence of reducing systems minimal. Hill & Scarisbrick (1940) used the initial rate of oxyhaemoglobin production as a measure of the initial rate of oxygen output during illumination in the chloroplast reaction. They assumed that over a short range haemoglobin combines with oxygen in direct proportion to its concentration.

With *Ascaris* haemoglobin, in presence of an active oxygen producing system (figure 1*a*), the initial rate of oxygenation is maintained up to complete saturation. The haemoglobins appear to combine irreversibly with oxygen in the manner of a true oxidation. But it has been shown previously (Davenport 1949) that the high oxygen affinity of the haemoglobins is the result principally of an extremely low velocity of dissociation of oxygen from combination with the pigments. The dissociation velocity, in presence of sodium hyposulphite, is independent of the concentration of the reducer. This, as was pointed out originally by Hartridge & Roughton (1923), is characteristic of the deoxygenations peculiar to the respiratory pigments. It was therefore concluded that the oxygen affinities of the *Ascaris* haemoglobins are so high that when they are subjected to successive small increments of oxygen concentration, a 5 % error in the method of measurement of the oxyhaemoglobin produced is too great to allow the effect of an equilibrium with oxygen to be detected. Moreover the effect of the oxygen uptake of the solutions upon the oxygenated haemoglobin also comes within the experimental error of the method.

In reactions where the initial rate of oxygenation of *Ascaris* haemoglobin is about one-third of that in figure 1*a*, indicating a less vigorous oxygen evolution, the rate of oxygenation diminishes exponentially with the time of illumination. For the reasons given above it is assumed that this diminution is the result of an

exponential decrease of oxygen production. This is always more rapid than that which occurs when chloroplasts are stored in darkness before use. Thus in a typical experiment the oxygen output fell to half in about 650 sec. illumination whereas 140 min. storage in darkness was required for the same loss of initial activity. A possible explanation of the observation is that exhaustion of some essential intermediate substance occurs. It is unlikely that the activity of the hydrogen acceptors present in the leaf extracts is limiting since variation in their concentration relative to the amount of chloroplast suspension added has no appreciable effect upon the rate of decrease of oxygen output although the initial rate is altered.

On the other hand, increased light intensity raises the initial oxygen output but also greatly accelerates the decrease in oxygen production during illumination. Hill & Scarisbrick (1940) investigated the effect of light intensity on the oxygen evolution of chloroplasts using the initial rate of oxygenation of muscle haemoglobin as a measure of the activity of the systems. They found that the initial oxygen output rose with increasing light intensity up to a point, at about 40,000 lux, where light saturation occurred. The occurrence of light saturation indicates that some process of limiting velocity participates in the photochemical reaction. Such a process would also explain the decline in oxygen evolution during illumination and its acceleration at high light intensities.

The comparative experiments with muscle haemoglobin serve to emphasize the extreme oxygen affinities of the *Ascaris* haemoglobins. The maximum percentage oxygenation attained was about one-tenth the maximum obtained by Hill (1939) yet under the influence of chloroplast systems of this low activity oxygenation of the *Ascaris* haemoglobins proceeded to full saturation. The use of these haemoglobins thus greatly increases the sensitivity of this method of estimating oxygen production. The limiting factor in their application is the difficulty in bringing about preliminary deoxygenation and for this reason they have not yet been applied to the measurement of oxygen in the more easily controlled ferric oxalate reaction of Hill (1939). An extension of their field of usefulness in the measurement of very small oxygen concentrations must await the finding of a hydrogen donator capable of bringing about deoxygenation without influencing the oxygen production of the chloroplasts.

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# Upper Ordovician trilobites of zonal value in south-east Shropshire

BY (THE LATE) B. B. BANCROFT; EDITED BY A. LAMONT

(Communicated by C. J. Stubblefield, F.R.S.—Received 20 July 1948)

[Plates 9 to 11]

The following descriptions of Shropshire trilobites, mostly collected from Murchison's (1839) type section of the Caradoc Series, are from Bancroft manuscripts which will ultimately be deposited in the British Museum (Natural History), where the Bancroft Collection of fossils is housed. As far as possible the original text has been preserved, apart from material necessary to bring it up to date and insertion of references to various publications. Since in Bancroft's paper on *Cryptolithinae* (1929) the illustrations were reproduced from drawings, it has been thought well, as suggested by Richter (1930, pp. 556–557), to refigure some of the species from photographs. A few of Bancroft's unpublished trilobites have been dealt with by Reed (1932) and Shirley (1936). In these cases only a minimum of reference is now given, but Bancroft's illustrations have been retained. A recent paper by Harper (1947) describes some related species from the Lower Longvillian of Caernarvonshire. It may be added that Dr C. J. Stubblefield has given help with the text of the present publication. He, Mr R. Baker and Mr W. H. C. Ramsbottom searched for the type specimens in the Natural History Museum. Thanks to their co-operation, most of the figured examples have now been identified with the photographs.

A.L.

## TRILOBITA

Family TRINUCLEIDAE Emmrich

Subfamily CRYPTOLITHINAE Bancroft, 1933, emend. Whittington, 1941a

Genus *Salterolithus* Bancroft, 1929

*Salterolithus* aff. *harnagensis* Bancroft

Figure 15, plate 10

This form, recalling *Salterolithus harnagensis* Bancroft from just above the Hoar Edge Grit (Bancroft 1929, pp. 80–81), is recorded from the Sowerbyella Grit as developed in fissures in Uriconian rocks, east of Church Stretton. It differs from *S. harnagensis* in having an extra pit in  $E_1$  on the median line in front of the glabella, as well as additional pits in  $E_2$  in the same position. An undescribed species, from the Spy Wood Grit, differs in more numerous and more widely spaced, intercalated pits in  $E_1$  and  $E_2$ , and has a single I-row of four pits in front of the glabella. See also Strachan, Temple & Williams (1948).

**Salterolithus smeathenensis** n.sp.

Figures 1, 2 and 2a, plate 9

**DIAGNOSIS**

Surface of glabella smooth at maturity, coarsely reticulate in early neanic stages. E-region of ventral fringe plate forming a flattened zone with four concentric rows of pits.  $E_1$  is a regular concentric row, similar to  $I_1$  but with somewhat smaller pits. Rows external to  $E_1$  have intercalated pits, and arrangement tends to be radial or quincuncial instead of concentric.  $E_2$  has intercalated pits mainly opposite antero-lateral margins of cheeks.  $E_3$  extends posteriorly to within 2 to 4 pit-radii from genal angles.  $E_4$  continuous, reaches posteriorly to, or nearly to, mid-cheek region. A few pits of  $E_5$  sometimes present in front. Between median line and genal angle about 96 pits in all in rows external to  $E_1$ .  $I_1$  and  $I_2$  continuous, similar.  $I_1$  slightly depressed between concentric, conspicuous ridges elevated above E-region.  $I_1$  with 22 to 26 pits (mode: 24).  $I_3$  extending anteriorly to within pit-radii 5 to 12 from pseudo-antennary pit.

Pygidium strongly arched mesially about an elevated rachis, concave on the sides between longitudinal folds and swellings in antero-lateral angles; margin rostrate at posterior extremity, with a well-defined lateral flexure. Axis strongly elevated with three strong rings, separated by sharply incised furrows; several less well-defined rings in posterior half. Pleurae with two or three segmental furrows, most deeply incised near margin.

**LOCALITY AND HORIZON**

Old cart track, 70 yd. north of extreme south-east corner of Smeathen Wood, Horderley, in sandy mudstones overlying the Hoar Edge Grit. Bancroft Collection: Horderley W5. *Reuscholithus reuschi* zone; Basal Harnagian.

**AFFINITIES**

*Salterolithus smeathenensis* resembles the contemporaneous *S. harnagensis* and *S. aff. harnagensis* in having a low  $I_1$  count and several distinct rings on the axis of the pygidium. It is also noteworthy that in the stratigraphical sequence *S. smeathenensis*, *S. cf. intermedius* (Wade), *S. caractaci* (Murchison), there is a progressive decrease in the modes for the  $E_2$  to  $E_5$  count. This is exhibited in the fifth column of table 1. The data for the  $I_1$  count suggest that the mode approximates to that in *S. caractaci*.

TABLE 1

species of <i>Salterolithus</i>	$I_1$			$E_2$ to $E_5$		
	L	M	H	L	M	H
<i>S. caractaci</i>	23	26-8	31	—	57-61	—
<i>S. cf. intermedius</i>	25	—	29	—	75-80	—
<i>S. smeathenensis</i>	22	24	26	94	96-97	99

The pygidium in *S. smeathenensis* with the strongly arched mesial zone is a primitive type found in the young of later *Salterolithi*. In these the mesial con-

vexity is less pronounced, the surface of the pygidium as a whole is much flatter, and the number of strongly defined rings on the axis is reduced. Most of these species are from more purely argillaceous sediments which have suffered to a greater extent from compression, but this cannot account for all the points of distinction.

**Salterolithus cf. intermedius** (Wade, 1911)

DIAGNOSIS

E<sub>3</sub> forming a continuous row extending to near the posterior extremities of the fringe. E<sub>4</sub> represented by a few pits in the pit-radii in the anterior region, or forming a short row. Zone external to E<sub>1</sub> from median line to genal angle with about 70 to 85 pits; mode about 75 to 80. I<sub>1</sub> with numerous pits; mode between 26 and 29 but further statistical work might narrow it down.

LOCALITIES AND HORIZON

In Bron-y-Buckley wood—Trilobite Dingle—Welshpool, in an exposure of red mudstone in the east bank of the stream (Bancroft Collection: Welshpool 41o). The same or a similar form occurs in the most southerly exposure in the stream section about 150 ft. north of the point where the stream meets the fence (Bancroft Collection: 41u); this is the lowest horizon in the section. The Horderley locality is in the track leading west from the stackyard at Glenburrell, 19 paces west of the hedge on the west side of the yard. All these localities are in beds underlying horizons with *Salterolithus caractaci* and above the *Reuscholithus reuschi* zone.

**Salterolithus caractaci** (Murchison, 1839)

*Trinucleus caractaci* Murchison, 1839, p. 659, plate xxiii, figures 1a, b, c (non d, e, f).

*T. concentricus* Murchison, 1872, plate iv, figures 2, 4, 5.

*Salterolithus caractaci* Bancroft, 1929, p. 78.

DIAGNOSIS

Glabella and cheeks smooth. Dorsal fringe plate flat. E-region with three well-defined concentric rows, or abnormally with the outermost row (E<sub>3</sub>) defective on the sides. Pits of E<sub>1</sub> form a regular concentric row, similar to or somewhat smaller than those of I<sub>1</sub> and with a few intercalated pits. E<sub>2</sub> continuous, the pits showing irregular radial, or quincuncial, arrangement with those of E<sub>1</sub> and E<sub>3</sub>, the fundamental radial disposition being modified by intercalations in E<sub>3</sub>. E<sub>3</sub> extends posteriorly to between mid-cheek region and posterior extremity of fringe, sometimes locally defective in antero-lateral regions. A few pits of E<sub>4</sub> sometimes present in the radii anteriorly. Concentric ridges in E-region locally irregular or depressed between stronger radial ridges. Norm of E<sub>2</sub> to E<sub>5</sub> count 57 to 61. I-region with two continuous rows of pits. I<sub>1</sub> feebly depressed, usually with 26 to 28 pits, between a pair of slightly conspicuous and elevated concentric ridges, somewhat stronger than the radial partitions, but becoming less prominent in front of glabella. Pits of I<sub>2</sub> similar to, or somewhat smaller than those of I<sub>1</sub>. I<sub>3</sub> extending anteriorly to within 16 to 8 pit-radii from the axial furrow. Ventral fringe plate with a strong

girder defining a flat E-region; I-region with two pseudo-girders in the postero-lateral region.

Pygidium with rounded, longitudinal lateral flexures, mucronate at posterior extremity. Axis depressed, with two or three strong rings, of which the first two are defined by two sharply incised grooves; rest of axis smooth, but with many discontinuous segmental divisions on inner surface. Lateral lobes nearly flat, obscurely arched about axis and with low swellings in the lateral angles; with two or three strong furrows externally, while internal mould has indications of several additional weaker ones.

#### LOCALITIES AND HORIZON

In the greenish sandstone of the stream section in Bron-y-Buckley wood—Trilobite Dingle—Welshpool, Montgomeryshire, in a good exposure quarried by the east side of the stream, about 240 ft. south of the north end of the Dingle, and nearly directly below an exposure in red mudstone by the path (Welshpool 41*h*). This locality is referred to as Welshpool 41*i*. The main Shropshire locality is in the plantation, about 700 ft. due north of Glenburrell, Horderley, and on the west side of the stream, in a digging 5 paces from the plantation fence and 59 paces from the hedge-fence junction to the south (Bancroft Collection: Y 51'). The locality Y 51 is not precisely located, but lies within a few feet of Y 51'. At both places the rock may be found at a few feet below the surface. The horizon is in the zone of *Salterolithus caractaci*.

#### ASSOCIATED FAUNA

At Horderley the assemblage includes small *Wattsella*, *Soudleyella* (s.l.), *Chonetidea*, *Cleidophorus*, an unnamed basal Harnagian lamellibranch, and graptolites including *Climacograptus*. At Welshpool the horizon yields the same unnamed lamellibranch and *Soudleyella* (s.l.).

#### AFFINITIES

Forms closely allied to *Salterolithus caractaci* occur at several horizons in the middle part of the Harnagian. The same group reappears a little above the base of the Soudleyan, and in the Shropshire and Welshpool areas is characteristic of the lower part of that stage. With the exception of specimens from Welshpool 41*h*, which have a low mode for the total of pits in the region external to  $E_1$ , these assemblages may be regarded as varieties of *S. caractaci*. Of less certain relationship are a group of forms from the *Broeggerolithus* burst and the subjacent horizon in the Soudleyan. These are essentially four-row forms with a few pits of  $E_3$  in front and more rarely on the sides. They seem to be closely related to the mid-Harnagian species from Welshpool 41*h*.

At the horizon of the *Horderleyella* burst at Horderley, there is a form in which  $I_3$  seems to extend farther anteriorly than in *Salterolithus caractaci* forma typica. If this proves to be the general rule in Soudleyan forms, it will be an easy means of determining the horizon.

Subgenus *Ulricholithus* Bancroft, 1933\*

## DESCRIPTION

The glabella is elongate but not swollen or very convex. It and the cheeks are smooth. The fringe has four continuous rows of perforations,  $E_2$ ,  $E_1$ ,  $I_1$ ,  $I_2$ . A discontinuous  $I_3$  row extends to between the mid-cheek region and the axial furrow.  $E_3$  is normally absent or restricted to a few pits on the front margin in a position between the pseudo-antennary pits.  $E_2$  usually shows intercalations on sides; in front, it is slightly depressed below the rest of the dorsal surface.  $E_1$  is somewhat elevated with feebly developed plates on the lateral radii.  $I_1$  is similar to  $E_1$ , but is depressed laterally, possessing 23 or 24 pits, rarely 25 or 26. As many as 6 but usually 3 or 4 pits of  $E_1$  are external at the posterior extremity of the fringe.

The pygidium resembles that of *Salterolithus*.

*Ulricholithus* may be regarded as a subgenus of *Salterolithus*, from which it is broadly distinguished by the absence of  $E_3$  except in front of the glabella, and by the low mode for the  $I_1$  count. *Ulricholithus* comes closest to *Salterolithus harnagensis* in which the counts for E and I have much the same range. There are, however, points of difference. (1) In *Ulricholithus* the radial ridges of E are elevated above the concentric ridge between  $E_1$  and  $I_1$ . This holds for all except a few radii near the posterior margin, and in some individuals is also true of these. In *Salterolithus harnagensis* radial and concentric ridges are almost equally developed except in front; on the sides radial arrangement is more or less completely replaced by quincuncial disposition, in which concentric ridges are angulated at junctions with radii and the latter are displaced sideways in alternate rows. This may be accentuated by intercalated pits in the concentric rows. (2) In some individuals of *S. harnagensis*  $E_2$  forms an irregular row of small pits, some of which are displaced outwards giving an incipient  $E_3$  row. On occasion a few well-defined pits of  $E_3$  may be present on the sides. (3) Early neanic stages of *S. harnagensis* have coarsely reticulate glabella and cheeks. In *Ulricholithus* at comparable stages they are smooth.

Though allied, *Ulricholithus* cannot be regarded as an early member of the genus *Broeggerolithus* which has reticulate ornamentation of the cephalon and in which stratigraphically early species are of notably smaller size than *Ulricholithus*. *Broeggerolithus* also has strongly elevated radial plates in  $E_1$  and smaller modal numbers for the  $I_1$  count. Apart from the reticulation, discrimination is sometimes difficult as in the case of an undescribed *Broeggerolithus* from the limestone band below the Frondderw Ash at Bala, where there seems to be convergence with a variety of *Ulricholithus*. In late large *Broeggerolithi*, like *B. longiceps* (Bancroft) from basal Upper Longivillian, the  $I_1$  modal number may be the same as in some *Ulricholithi*. In such cases, criteria for distinction for *Ulricholithus* include less swollen and elevated glabella and absence of reticulation.

\* Doubtfully valid in 1933 in terms of Article 25 of the International Rules of Zoological Nomenclature since the type species cited was at that time undescribed.—C. J. Stubblefield.

## STRATIGRAPHICAL RANGE

Late Harnagian and Basal Soudleyan.

## SUBGENOTYPE

*Ulricholithus ulrichi* Bancroft.

**Ulricholithus ulrichi** Bancroft, 1933, name only

Figure 14, plate 9

*Ulricholithus ulrichi* Bancroft, 1933, p. 2 and table 1.

## DIAGNOSIS

Cephalon 22 to 29 mm. wide, semicircular or with a slight constriction in front of the genal angles which are only a little posterior to the nuchal segment. Glabella elongate. Dorsal fringe plate with strong radial and concentric arrangement in the three outermost rows ( $E_2$ ,  $E_1$ ,  $I_1$ ) except in front of the glabella, or the radial arrangement may be modified locally by intercalations in  $E_2$ .  $E_3$  absent, or rarely represented by 2 or 3 pits in front of glabella.  $E_2$  with 20 to 26 pits, apparent mode 24, slightly depressed on the sides, with 0 to 4 intercalations laterally and 1 to 7 in front, extending to within 2 to 6 pit-radii from the genal angle.  $E_1$  similar to  $I_1$ , slightly elevated above  $E_2$  and  $I_1$ , with elevated radial plates on sides or the radial ridges rounded. E-region sometimes with a large intercalated pit in front of the glabella.  $I_1$  with 23 to 25 pits, a not sharply defined mode on 23, slightly depressed at sides, and often deflected outwards towards the genal angles posteriorly.  $I_2$  bounded externally by a strong concentric ridge on the sides, with somewhat smaller and more numerous pits than  $I_1$ .  $I_3$  on each side extending anteriorly to between 6 and 10 pit-radii from the axial furrow. Ventral fringe plate with a well-defined girder and two pseudo-girders (between  $E_1$ - $I_1$  and  $I_1$ - $I_2$ ); pseudo-girder between  $E_1$  and  $I_1$  similar to the girder;  $I_1$  depressed.

Pygidium less transverse than in normal *Salterolithus*. Axis with two strong rings and articular ring, separated by sharply incised grooves, smooth or feebly segmented posteriorly. Pleural lobes with 2 or 3 pairs of furrows, each with a transverse rib, or ribs obsolete except near margin. Interior of rachis clearly segmented through about two-thirds of its length.

## LOCALITIES AND HORIZON

Stream section in Middle House dingle, 120 yd. west-north-west of Middle House, north of Welshpool (Bancroft Collection: Welshpool 1b; also 20 ft. west is locality 1c5, and immediately above it 1d, the locality from which the specimen is figured. Near base of *Ulricholithus ulrichi* zone. Copse section, 280 yd. north-west of Middle House, and almost one-third of a mile north of the Navigation Inn (Welshpool 1l),\* about 300 ft. higher in section than locality 1b. Shropshire localities are in the large exposure by the north side of the cartway, north-west of Glenburrell, and 70 to 86 ft. north-east of the stackyard gate, in basal mudstones of the Glenburrell Beds with 'ash balls' (Bancroft Collection: Horderley Y24a);

\* Bancroft suggests intercalations in  $E_2$  on the sides have a mode of 3 or 4 for this locality, but apparently he only made up complete statistics for 1b, 1c and 1d forms.—A.L.

also about 60 ft. south-west of hedge-junction, in north side of cartway, at the summit of the nearly continuous series of mudstones with hard flaggy bands (Horderley Y 24d).

#### ASSOCIATED FAUNA

At the type locality (Welshpool 1d), *Cleidophorus* sp. and *Sinuities* sp. occur, but no Dalmanellacea. At Welshpool 1b there are graptolites. As also may be indicated by the absence of reticulation, this suggests that *Ulricholithus* belonged to moderately deep muddy water.

#### Genus *Reuscholithus* Bancroft, 1929

##### *Reuscholithus reuschi* Bancroft, 1929

Figure 3, plate 9

*Reuscholithus reuschi* Bancroft, 1929, pp. 82–85, text-figures 4a–b, plate i, figures 3, 4 and 5a–b.

#### Genus *Marrolithus* Bancroft, 1929

##### *Marrolithus ultimus* n.sp.

Figure 7, plate 9

#### DIAGNOSIS

Head shield strongly convex, with swollen cheeks and steeply inclined fringe. Antero-lateral ‘angulations’ rounded, lateral margins slightly concave to nearly straight. Ventral fringe plate with very broad and strong girder in front but losing distinctive character in antero-lateral region.  $E_1$  row continuous,  $E_2$  consisting of a few pits in front of glabella.  $I_1$  and  $I_2$  continuous, rather large pits,  $I_1$  with 23 to 26 pits.  $I_3, I_4, I_5, I_6$  discontinuous.  $I_3$  extending to glabella,  $I_4$  extending to within third or fifth pit-radius from axial furrow.  $I_6$  represented by a few pits on side of cheek. Posterior pit-complex indefinite, with more or less regular concentric and radial arrangement. About 11 pits along posterior margin of fringe.

Pygidium subarcuate, without lateral flexure. Axis with two strong segmental furrows; pleural lobes with only obscure traces of segmentation. Otherwise smooth.

#### LOCALITY AND HORIZON

In the quarry, 500 ft. south of Coston farmhouse, south of Aston-on-Clun, in the highest 2 ft. exposed (Bancroft Collection: Coston 21c5). The rock is a yellowish brown grit, a few feet above beds with *Smeathenella strophomenoides* Bancroft, and lies below the *Dinorthis* (*Plaesiomys*) *robusta* subzone. Zone of *Horderleyella plicata*.

#### AFFINITIES

Genal spines, external spine channel corresponding with  $E_1$ , and nuchal spine are as in other *Marrolithi*. Mode for  $I_1$  count seems to be 25 to 26, but statistics may be faulty owing to the coarse nature of the matrix.

The Llandeilian *Marrolithi* include *M. favus* (Salter) and *M. bureaui* (Oehlert). Both these species have a single row of pits in the region external to the girder. The Lower Costonian of Chirbury and east Shropshire provides undescribed species

with less angular cephalala and I-regions of reduced complexity as compared with the Llandeilian forms. Towards the summit of the Costonian, species like *M. ultimus* show a trend to increased complexity of the I-region, and at the same time a few pits of  $E_2$  appear in front of the glabella.

Extreme reduction of the I-region and tendency towards antero-lateral angulation of the fringe margin are found in *Eirelithus thersites* (Salter) from the Tramore Limestone, stage 3 (Llandeilo-Caradoc) (Lamont 1941). It has complete  $E_1$  and  $E_2$  rows, and sometimes  $E_3$  antero-laterally.

#### Genus **Broeggerolithus** Stubblefield in Lamont, 1935

##### **Broeggerolithus broeggeri** (Bancroft, 1929)

Figures 4 and 5, plate 9

*Cryptolithus broeggeri* Bancroft, 1929, pp. 85–86, plate i, figures 6–8.

*Broeggeria broeggeri* Bancroft, 1933, p. 2.

##### **Broeggerolithus soudleyensis** (Bancroft, 1929)

Figure 6, plate 9

*Cryptolithus soudleyensis* Bancroft, 1929, pp. 86–88, text-figures 5a–b, plate i, figures 9–11.

*Broeggeria soudleyensis* Bancroft, 1933, table 2.

##### **Broeggerolithus transiens** (Bancroft, 1929)

Figure 8, plate 9

*Cryptolithus transiens* Bancroft, 1929, pp. 90–92, plate ii, figure 5.

##### **Broeggerolithus constrictus** n.sp.

Figure 38, plate 9

#### DIAGNOSIS

This group is characterized by having three pits of  $E_1$  external towards the genal angle, by an abrupt constriction of the fringe behind the most posterior pit-radius with both  $E_1$  and  $E_2$ , and by the unusual triangular contour of the pygidium.

#### LOCALITIES AND HORIZON

The more important type locality is in the stream from Ceunant to below Cockshut, in the Welshpool district. The exact position is 515 yd. west-south-west of Ceunant and 80 ft. east of the hedge at the west end of the Mill Pond, in tough greenish grey mudstone (Bancroft Collection: Welshpool 51e). The Shropshire position is defined with reference to locality P 10, in *Broeggerolithus broeggeri* shales, in a small stream at the extreme north-east end of Smeathen Wood. An index point is taken in the Onny, on a line perpendicular to the bank of the Onny and running through P 10. Eight yards east of this index point *Broeggerolithus constrictus* n.sp. occurs along with brachiopods and *Lophospira*. Seven paces west of the index point and 4 paces west of a fence by the stream, normal *B. broeggeri*



is abundant. The *B. constrictus* locality is numbered P110g in the Bancroft Collection; the *E. broeggeri* locality is P110c.\*

As shown by the author in his 'Correlation Tables' (Bancroft 1933), the *Broeggerolithus constrictus* beds are at the base of the Soudleyan at Welshpool. This horizon is immediately above the zone of *Ulricholithus ulrichi*.

Genus **Onnia** Bancroft, 1933

**Onnia cobboldi** (Bancroft, 1929)

Figures 10 and 11, plate 9

*Cryptolithus cobboldi* Bancroft, 1929, pp. 92-94, plate ii, figures 6 and 7.

*Onnia cobboldi* Bancroft, 1933, table 1.

*O. cobboldi* Lamont, 1948, p. 418, plate i, figures 6-8.

**Onnia gracilis** (Bancroft, 1929)

Figure 9, plate 9

*Cryptolithus gracilis* Bancroft, 1929, pp. 94-95, plate ii, figures 8 and 9.

*Onnia gracilis* Bancroft, 1933, table 1.

*O. gracilis* Lamont, 1948, p. 418, plate i, figure 9.

**Onnia superba** (Bancroft, 1929)

Figure 12, plate 10; figure 16, plate 10

*Cryptolithus superbus* Bancroft, 1929, pp. 95-96, plate ii, figure 10.

*Onnia superba* Bancroft, 1933, p. 2.

*O. superba* Lamont, 1948, p. 418, plate i, figures 1-5.

**Onnia superba** n.var.?

Figure 13, plate 9

DIAGNOSIS

A possible variety occurs as a rare associate of *Onnia superba*. It differs in that  $I_3$  extends to in front of the axial furrow.  $I_4$  has more numerous and larger pits than in typical *Onnia superba*, and extends to the fifth pit-radius of  $I_2$  from the axial furrow. In large *O. superba*, however, it may extend to the seventh.

Family RAPHIOPHORIDAE Angelin

Genus **Lonchodomas** Angelin

**Lonchodomas pennatus** (La Touche), revised by Reed, 1932

Figure 17, plate 10

AFFINITIES

The present figure emphasizes the slight carination of the glabella, but this feature is not so marked as in the allied *Lonchodomas* aff. *rostratus* (Sars) as

\* Diagrams of localities are being prepared; but only that of the Actonian-Onnian of the Onny section can be given with the present paper (see figure 39). Many of the collecting localities, but not all, are defined in the Bancroft Catalogues at the British Museum (Natural History).—A.L.

illustrated by Størmer (1945, plate ii, figure 10) from the Tretaspis Shale (?) of Hadeland. For a more extreme development of a carinal ridge, compare *Ampyx* (?) *aculeatus* Angelin (Thorslund, 1940, plate 9, figure 11).

#### LOCALITY AND HORIZON

In beds with *Onnia gracilis*, 11 to 22 ft. below the base of the *O. superba* shales, where there is a low ledge of calcareous mudstone in the Onny River, about 40 yd. west of the west end of the 'Cliff' section, near Wistanstow. Bancroft Collection: Horderley Pc (see figure 39).

#### Genus **Raphiophorus** Angelin

#### **Raphiophorus edgelli** Reed, 1932

Figure 18, plate 10

#### LOCALITIES (?) AND HORIZON

Reed (1932) reported this species from 'Horderley Quarry' and the Onny River. The present pygidium is from Acton Scott Beds with *Reuschella semiglobata* Bancroft, at Jack Slither, in a hard calcareous band about 4 in. thick and 15 ft. below the highest beds exposed (see figure 39).

#### Family REMOPLEURIDIDAE Hawle & Corda

#### Genus **Remopleurides** Portlock

#### **Remopleurides burmeisteri** n.sp.

Figures 19 and 20, plate 10

#### DIAGNOSIS

Glabella between eyes transversely elliptical, with a narrow, moderately long anterior tongue, slightly bent down in front. Sides of tongue nearly parallel. Three pairs of glabellar furrows, with inner ends equal distances apart; anterior pair short, faint, slightly convex to the front, and directed obliquely backwards; second pair sharply incised, long, gently convex forwards, less oblique than first pair; third pair similar to second but still less oblique, much more strongly curved, with their inner ends slightly concave to front. Axial and neck furrows narrow, sharply incised. Palpebral lobes semicircular, closely applied to sides of glabella, narrow, except against fourth glabellar segment, in front of which they are constricted and thence taper more gradually in an anterior direction. Eyes forming low bands of minute lenses, borne on a fine, low, semicircular platform rising abruptly from the cheek. Free cheeks long and narrow, with wide and deep furrows running inside the lateral margins; inner edges of furrows terminating against middle of eye. Behind this point and inside the furrow there is a small triangular area. Genal angles produced in long, narrow genal spines.

Axis of thorax only moderately wide for the genus, with straight sides converging slightly posteriorly (only first six segments known), without median spines or tubercles. Pleurae rather less than two-thirds the width of axis, with lateral extremities slightly oblique, each produced backwards in a short spinous projection

—the segments becoming somewhat longer and more spinous posteriorly. Pleural furrows strong, oblique.

Pygidium unknown.

#### DIMENSIONS

	Two examples (mm.)	One example (mm.)
Length of glabella	7	7.25
Width of glabella	8	9.25
Width of tongue at base	3.75	3.75

#### AFFINITIES

This species is distinguished from *Remopleurides barrandei* Etheridge jnr. & Nicholson and *R. correctus* Reed by the much smaller tongue of the glabella and the more oblique glabellar furrows. The tongue is less transverse than in *R. colbii* Portlock, *R. longicostatus* Portlock, *R. salteri* Reed, and *R. nanus* (Leuchtenberg), but rather more transverse than in *R. wimani* Thorslund. The axis of the thorax is somewhat wider than in *R. dorsospinifer* Portlock and *R. colbii*, and much narrower than in *R. salteri*. In relative lengths the glabellar furrows compare with those in *R. latus* var. *granensis* Størmer (1945, plate iv, figure 4) from the Gagnun Shale, but are much more oblique. *Caphyra* cf. *radians* Barrande, as found in the Ashgillian at Pwllheli (Matley, 1938), also has oblique furrows, but a smaller tongue.

#### LOCALITY AND HORIZON

Onny Shales, in the 'Cliff' section in the north bank of the Onny River. See figure 39. *Onnia superba* zone.

### Family ODONTOPLEURIDAE Burmeister

#### Genus *Acidaspis* Murchison

#### *Acidaspis harnagensis* n.sp.

Figures 21 and 22, plate 10

#### DIAGNOSIS

Glabella with three pairs of lateral lobes; the first (anterior) pair very small, each separated from the middle lobe by a well-defined groove; second glabellar lobes oval, elongate from anterior to posterior, less than half as long as the large third (basal) lobes. Neck-furrow clearly defined. Neck-ring long, narrowing posteriorly; oblique grooves parallel with and close to sides, confluent with nuchal furrow behind basal lobes; ill-defined median tubercle in front of slight emargination. Fixed cheeks prominent, with narrow ocular ridges extending from front of glabella to opposite middle of third lateral lobes, with fine groove along inner side. Axial furrows deep and narrow. Free cheeks unknown. Surface of middle shield covered with closely set tubercles.

Pygidium broad and short, a little more than three times as wide as long, excluding spines. Axis strongly convex, only slightly tapering posteriorly; anterior half ring for articulation unknown, followed behind by one short, elevated ring

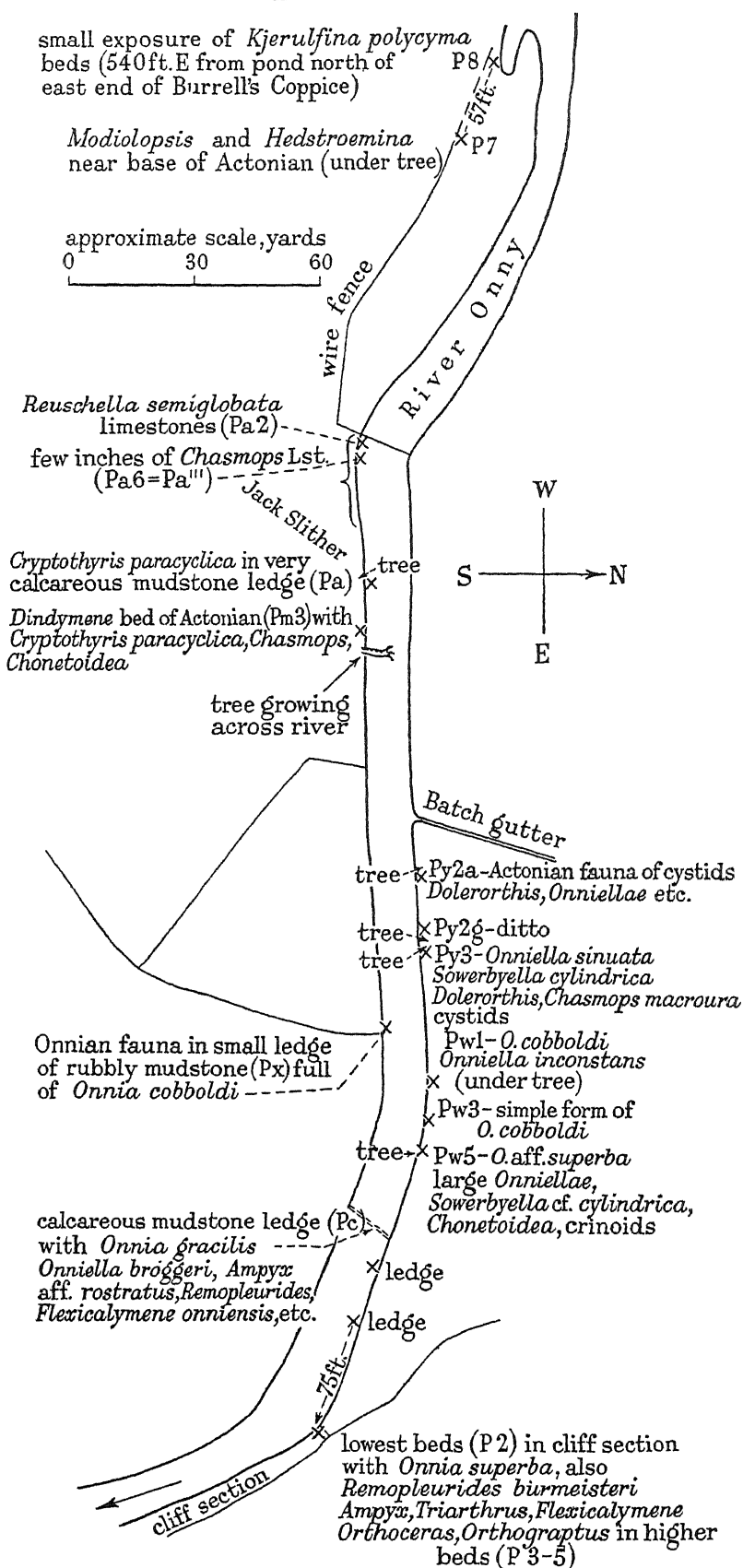


FIGURE 39. Diagram of Actonian and Onnian fossiliferous localities in the River Onny, near Wistanstow.

and two longer, lower rings separated from the first by a deep, wide furrow and from one another by a less conspicuous furrow. Lateral lobes flattened, traversed by strong elevated ridge arising from the first axial segment and continued over the border as the fifth spine. A marked groove running parallel with the sides from the front to near the base of the oblique ridge, confluent with a wide groove extending along the outer side of the ridge and separating a wide border and an inner triangular area. Border of pygidium with seven pairs of straight spines; first pair arising from antero-lateral angles, very short; second, third, and fourth pairs stouter than first, long; the third longer than the second and apparently the fourth longer than the third. Fifth pair of spines very stout, much longer than the others, continuous with oblique ridges. Sixth and seventh pairs of spines about as stout as fourth. Surface of pygidium tuberculate, tubercles numerous on axis, few and scattered on lateral lobes, about twenty-seven on inner part of lateral lobe external to ridge, and seven on border. Furrow on outer side of oblique ridge smooth.

#### LOCALITY AND HORIZON

In sides of old cartway, about 70 yd. north of extreme south end of Smeathen Wood, Horderley, in sandy mudstone a few feet above the Hoar Edge Grit. Bancroft Collection: Horderley W5. Basal Harnagian; *Reuscholithus reuschi* zone.

#### AFFINITIES

This species seems sufficiently distinct from *Acidaspis caractaci* Salter (1853, p. 7), from Gretton Quarry, near Cardington, which is stated to have two pairs of glabellar lobes and six terminal spines between a divergent very strong pair on the pygidium. The Smeathen Wood species is distinguished by having the ridge on the lateral lobes of the pygidium continuous with the fifth pair of spines. This is also the case in *Onchaspis hystrix* (Wyville Thomson), which, however, shows less differentiation in lengths of spines. In *Acidaspis coronata* Salter and *A. dalecarlica* Törnquist the transverse ridges are continuous with the second and third pairs of spines respectively, and the total number of spines is different. The long tuberculate occipital ring recalls that of *Onchaspis lalage* (Wyville Thomson), but paired nuchal spines have not been observed as in the Balclatchie species.

? Family PROETIDAE Hawle & Corda

#### *Proetidella* n.g.

#### DEFINITION

*Proetidella* differs from *Proetus* in three main respects: (1) The cephalon is surmounted at front and sides by a very thin marginal rim. This rim is continued down the outer sides of the genal spines. Within the rim is a wide depressed area, horizontal at the sides and inclined in front, with a shallow angular groove running round its proximal margin and separating the shovelling (?) border from the slightly convex inner part of the cheek. The homologies are not clear, but something of the same kind is found in the Ogygiocarinae of Raymond, for example in *Ogygiocaris buchii* (Brongniart). If the fine rim corresponds with the border in *Proetus*, then both the depressed band as well as the inner furrow must represent

the marginal furrow of that genus. The border, however, may be represented by both the rim and the wide depressed band. Reed figures a comparable border in *Proetus* (*Warburgella*) *stokesi* (Murchison) (Reed 1904, plate xi, figure 10). (2) The eyes are very long, closely applied to the sides of the glabella, and reach back to, or nearly to, the occipital furrow. (3) The pleurae of the pygidium are strongly curved backwards. There are four grooved pleural segments separated by strong furrows, the posterior margin of each segment elevated above, and appearing to overlap the front of the one behind. The fusion of the pleurae is ill marked; and the anterior one in the pygidium is not readily separable from those of the thorax. Flattened smooth triangular areas without segmentation, on either side of the posterior tip of the axis, are somewhat reminiscent of similar features in what Salter termed the female of *Ogygiocaris buchii* (1866, plate xv, figure 6).

#### GENOTYPE

*Proetidella fearnsidesi* n.g. et n.sp.

#### ***Proetidella fearnsidesi* n.g. et n.sp.**

Figure 23, plate 10

#### DIAGNOSIS

Head shield nearly two and a half times as wide as long, evenly curved at front and sides, with wide flat border, inclined in front of glabella and bounded externally by a fine rim. Marginal furrow shallow. Glabella short, expanded between eyes, narrower and tapering in front; frontal outline subangular. First two pairs of glabellar furrows obsolete, third pair typically fine and shallow. Outline of base of glabella slightly concave in middle. Narrow raised band uniting fixed cheeks in front of glabella. Axial furrows fine, uniting in front of glabella. Occipital furrow deeply incised, narrow, gently arched forward at sides and middle. Occipital ring strong, no median tubercle. Fixed cheeks show extreme reduction posteriorly, but widen rapidly in front of eyes. Palpebral lobes very long, extending from axial furrow forward along about three-fifths of length of glabella. Facial sutures cutting posterior margin very close to axial furrows, widely diverging in front of eyes. Free cheeks large, with marked posterior marginal furrow; proximally flattened genal spines extend to the fifth thoracic segment. Eyes close to glabella, large, crescentic, extending backwards to neck furrow; a wide groove surrounds their base against cheeks.

Thorax with ten segments. Axis moderately convex, tapering, somewhat wider than pleurae, with rings slightly arched forward in the middle and at the sides, without tubercles. Pleurae flattened, distal ends curved backwards and slightly inclined downwards; extremities sharply pointed.

Pygidium semicircular. Axial lobe tapering, strongly convex with six segments and articulating half-segment in front, also a small unsegmented posterior part, and a somewhat elevated broad ridge extending to margin behind. Lateral lobes with four weakly fused segments, each traversed by furrow reaching to margin.

The segments are strongly curved backwards, and their elevated posterior margins give rise to an imbricate appearance.

#### LOCALITY AND HORIZON

In the sides of the old cartway about 70 yd. north of the extreme south end of Smeathen Wood, Horderley, about 10 to 20 ft. above the top of the Hoar Edge Grit. Bancroft Collection: Horderley W5. Basal Harnagian, *Reuscholithus reuschi* zone.

#### Family CALYMENIDAE Milne Edwards

##### *Flexicalymene acantha* n.sp.

Figures 29, 30, plate 11

#### DIAGNOSIS

Glabella strongly convex, moderately long, relatively wide across basal lobes and narrow in front. Frontal lobe fairly long, convex anteriorly and at sides, tapering only a little. First pair of lateral lobes small, extending somewhat beyond lateral margin of frontal lobe. Second pair of lateral lobes large, transverse, directed obliquely forwards. Width across second pair conspicuously greater than across first pair, but only slightly less than across basal lobes. Third pair (basal) lobes quite large, very oblique in front. Fixed cheeks with palpebral lobes opposite the second pair of lateral lobes. Border in front moderately wide, outline slightly convex, upturned, fairly high, steeply inclined, separated from front of glabella by a sharply defined horizontal field. Neck-furrow deep, rather narrow. Axial furrows deep, wide opposite frontal and first lateral lobes, contracted opposite second and third lateral lobes. Surface of glabella and neck-ring covered regularly with numerous prickly tubercles; similar but more densely crowded spiny tubercles on border in front, more sparsely distributed on cheeks.

Pygidium (apparently of the same species) strongly arched in front, less strongly behind; axis narrow, much less than width of pleural lobe, strongly convex, with anterior half-ring followed by six rings all separated by strong furrows; a posterior undivided part; pleural lobes each with six segments separated by strong furrows and—except for half-segment in front—grooved throughout. Axial furrows strong.

#### LOCALITY AND HORIZON

Sides of old cartway, about 70 yd. north of the extreme south end of Smeathen Wood, Horderley, 10 to 25 ft. above Hoar Edge Grit. Bancroft Collection: Horderley W5. Basal Harnagian, *Reuscholithus reuschi* zone.

#### AFFINITIES

*Flexicalymene acantha* n.sp. is like some of the early stages of *F. senaria* (Conrad) as described by Whittington (1941*b*, plate 72, figure 14), but the glabella is less plump and the anterior border has a less curved outline than in *F. senaria* of comparable size. The border is much more steeply upturned than in *F. cambrensis* (Salter), the pygidium of which, however, is very like that of *F. acantha*, apart from the fact that it has more numerous segments. The prickly tubercles are relics of a neanic spinose condition, and may also be interpreted as a primitive bio-character.

Shape of the glabella suggests comparison with *Pharostoma*, but in the latter the first lateral lobes are more or less obsolescent and the horizontal pre-glabellar field is usually longer.

***Flexicalymene trigonoceps* n.sp.**

Figures 31, 32, 32*a* and 32*b*, plate 11

DIAGNOSIS

Head shield semicircular, twice as wide as long. Glabella moderately convex, rather short, wide at base, narrow in front; frontal lobe small, sides tapering, front straight. First pair of lateral lobes small, extending just beyond base of frontal lobe at sides; shallow, transverse furrow in front, not separated from middle part of glabella by a definite furrow. Second pair of lobes are globular, divided from first pair by deep and wide furrows and from middle region by a short depressed neck; they protrude beyond first pair laterally. Third pair of lobes subcuboidal, attached to glabella by narrow depressed neck, deeply incised furrow on inner sides, about twice as large as second pair and protruding beyond them at the sides. Surface of glabella fairly coarsely tuberculated.

Fixed cheeks gibbous with sharply bevelled inner edges, narrowest between eyes which are opposite the second lateral lobes, widening in front of eyes; high, wide, and long, inclined border in front. Neck-furrow deep and moderately wide behind glabella, very wide on cheeks. Axial furrows deep and wide, wider in middle than behind, or of uniform width, only slightly shallower in middle than posteriorly.

Pygidium subtriangular; sides steeply arched. Axis evenly convex at front and middle, dorsally flattened behind, with five strong rings and anterior articulating half-ring. Pleural lobes with five segments and anterior half-segment, separated by strong furrows, grooved through part of length. Axial furrows deep, continuous round rear end of axial lobe.

LOCALITIES AND HORIZON

At top of Old Quarry, and also in Lane section, in Marsh Wood, about  $\frac{1}{2}$  mile south of Marshbrook railway station, in silts with *Wattsella unguis* (J. de C. Sowerby). Middle Marshbrookian (near top of Longville Flags).

AFFINITIES

The glabella is longer, less flattened in front, and less wide across the basal lobes than in *Flexicalymene planimarginata* (Cowper Reed), as refigured by Shirley (1931, plate ii, figure 1). The border is less steeply inclined than in *F. caractaci* (Salter). In *F. jemtlandica* Thorslund, the outline of the anterior margin of the border is straight (1940, plate xii, figure 19), not crescentic.

***Flexicalymene salteri* n.sp.**

Figures 34, 34*a* and 35, plate 11

DIAGNOSIS

Glabella moderately convex, trigonal, wide at base, narrow in front, wider than cheek; frontal lobe small with sides converging anteriorly; front slightly convex. Width across first lateral lobes a little greater than that of frontal lobe; first lateral



lobes small; second pair larger, globular, attached to middle region of glabella by a short depressed neck, about half as large as third pair, third lateral lobes subquadrate-globular, joined to base of glabella by narrow depressed neck, short wide furrow separates inner side of each lobe from axial region of glabella. Fixed cheeks, only moderately elevated, form a wide, sloping, upturned border in front of glabella. Occipital furrow deep and fairly wide behind glabella; posterior marginal furrow wide, but only moderately deep, on cheeks. Axial furrows deep and narrow, narrowest opposite basal lobes; no buttress.

Glabella ornamented with irregular tubercles, the largest in the middle region. Cheeks covered with very numerous, closely set minute granules or tubercles of regular size.

#### LOCALITIES AND HORIZON

Small exposure in wood west of Rose Villa, Marshbrook, in calcareous mudstone. Acton Scott Beds with *Reuschella semiglobata* Bancroft. The same species occurs in highly calcareous shale and beds of limestone, in the Onny River at Jack Slither, on the north side of a field, east of the railway bridge, east of Burrell's Coppice.

#### AFFINITIES

The glabella is broader than in *Flexicalymene caractaci* as given by Salter in his supposedly generalized figure (1865, plate ix, figure 3), but Shirley's figures (especially 1931, plate ii, figure 6) come very close to the glabellar form of *F. salteri*, though they show the fixed cheeks more elevated than in figure 34 of the present paper. Unfortunately the horizon of *F. caractaci* in the Actonian is not precisely known. Its anterior border seems narrower in a transverse direction than that of *F. trigonoceps*. The latter has also coarser tuberculation, it is thought.

#### *Flexicalymene laticeps* n.sp.

Figures 33, 33a, plate 11

#### DIAGNOSIS

Glabella inflated, subtrigonal, narrow in front, width at base exceeding length. Frontal lobe elevated with vertical front. First lateral lobes very small, ridgelike, freely projecting beyond sides of frontal lobe, only a slight groove in front; second pair globular, separated from axial region by constriction and furrow; third pair subquadrate, joined to axial region by very narrow neck posteriorly; oblique branch of third glabellar furrow extending nearly to neck-furrow. Posterior end of glabella high, rising nearly vertically above narrow, deep neck furrow.

Fixed cheeks with palpebral lobes fairly far back, opposite the second and anterior part of the third lateral lobes. Border in front moderately broad, greatly depressed beneath top of glabella, without an inner horizontal zone, short from back to front. Posterior marginal furrow wide on cheeks, less deep than occipital furrow. Axial furrows deep, narrow opposite basal lobes, wider in front.

#### LOCALITY AND HORIZON

Small exposure near the extreme north end of the small wood, west of Rose Villa, Marshbrook, in calcareous mudstone. Acton Scott Beds with *Reuschella semiglobata*.

## AFFINITIES

The profile of this species (figure 33a) suggests comparison with that of *Flexicalymene quadrata* (W. B. R. King), but the anterior part of the glabella rises by no means as high above the anterior border. *F. brevicapitata* (Portlock) is also comparable, but has shorter glabella, wider hollow field between frontal lobe and upturned anterior border, and the palpebral lobes are opposite the front part of the second glabellar lobes.

**Flexicalymene onniensis Shirley, 1936**

Figures 36 and 37, plate 11

*Flexicalymene onniensis* Shirley, 1936, pp. 405-407, plate xxix, figures 5-7.

## LOCALITY AND HORIZON

In a ledge of calcareous mudstone running across the River Onny about 40 yd. west of the west end of the 'Cliff' section, near Wistanstow. *Onnia gracilis* zone. See also Lamont (1948).

## ASSOCIATED FAUNA

*Onnia gracilis* (Bancroft), *Lonchodomas pennatus* (La Touche), *Remopleurides* sp. and *Onniella broeggeri* Bancroft, which may be collected from 10 to 25 ft. below the base of the Onny Shales with *Onnia superba*. Bancroft Collection: locality Pc (see figure 39).

## AFFINITIES

The profile is not unlike that of *Flexicalymene laticeps*, but the glabella is narrower posteriorly and not trigonal in outline. The surface ornament is more regularly arranged than in *F. salteri*. In plan the outline of the anterior border is less arcuate than in *F. senaria* (Conrad). The front of the glabella also rises more steeply and the palpebral lobes are further back than in *F. senaria*; but the pygidia of the two species are similar. Both may be in lines of descent that may include *F. acantha* and *F. laticeps*.

Genus **Diacalymene** Kegel**Diacalymene praecox** n.sp.

Figures 28 and 28a, plate 11

## DIAGNOSIS

Glabella strongly convex, very long, extending almost to inner margin of thickened border, wide across basal lobes, much narrower in front and tapering only slightly. Frontal lobe very long, sides gently convex, not converging anteriorly, nearly straight in front, almost as wide as width across first pair of lateral lobes. First pair of lateral lobes fairly large, hemispherical, not separated from axial region by groove or constriction; second pair less than twice the length of the first, globular, connecting with axial region by ill defined neck; third pair relatively very large, protruding laterally considerably beyond second pair. Fixed cheeks

not widening in front of palpebral lobes, separated by deep depression from nearly straight, almost vertical border with posterior side concave opposite the front of glabella and undercut beneath posteriorly sloping bevel. Axial furrows deep, very narrow, form a broad curve round the basal and second lobes, and form a less conspicuous curve round the sides of the frontal lobe. Surface covered with numerous small tubercles.

#### LOCALITY AND HORIZON

Sides of old cartway, about 70 yd. north of the extreme south end of Smeathen Wood, Horderley, 10 to 25 ft. above the Hoar Edge Grit. Bancroft Collection: Horderley W5. *Reuscholithus reuschi* zone.

#### AFFINITIES

It is very like *Diacalymene marginata* Shirley (1936, p. 391, figure 2, and plate xxix, figure 19) from the Lower Drummuck Group, Girvan, from which it differs in smaller size, in proportionately larger frontal lobe of the glabella, and in absence of irregular minor concavities in the outline of the posterior margin of the upturned and thickened anterior border. It seems to be in the same line of descent with *D. drummuckensis* (Reed) and *D. diademata* (Barrande). The relative width of the glabella between the basal lobes may come nearest that in *D. crassa* Shirley, from the Lower Llandovery, which is distinguished by having the bevelled surface on the anterior border approximately horizontal and not tilted backwards. *Reacalymene pusulosa* Shirley, though related, has a broad trigonal glabella. One of Størmer's Calymenids (1945, plate ii, figure 7) may be an allied *Diacalymene*.

*Diacalymene praecox* is important as anticipating an Upper Bala (Ashgillian) form. Another example of such anticipation has been noted at Girvan, Scotland, by Mr R. P. Tripp. In collecting from the lowest transgressive mudstones at Craighead Quarry, he has found a *Pterygometopus* apparently identical with *P. retardatus* Reed, which has been recorded from the Upper Drummuck Group (Cowper Reed, 1913, plate viii, figures 5-7; Lamont, 1947, figure 2).

#### Genus *Brongniartella* Cowper Reed

#### *Brongniartella horderleyensis* n.sp.?

Bancroft believed that this species with typically 10 rings on the axis of the pygidium should be distinguished from *Brongniartella rudis* (Salter) with 8 and *B. bisulcata* (Salter) with 12. The young cranidium of *B. horderleyensis* n.sp., he says, agrees with that of *B. rudis*. On the pygidium *B. horderleyensis* has six pleural segments, plus the articulating half segment in front. *B. parva* Harper has similar pleural segments, but fewer axial rings.—A.L.

#### LOCALITY AND HORIZON

Quarry by west side of New House, Horderley, a few feet below the base of the *Heterorthis alternata* phase. High level in Horderley Sandstone (Lower Longvillian).

## Family PHACOPIDAE Hawle &amp; Corda

Subfamily ACASTINAE Delo, 1935

**Phacopidina** n.g.

## DEFINITION

*Phacopidina* is proposed for species formerly referred for the most part to *Acaste*, but which differ from that genus as follows:

(1) The third pair of glabellar furrows are directed obliquely outwards and forward, so that the basal lobes form a pair of triangles with apices opposed. In *Acaste* the third pair of furrows are directed somewhat obliquely backwards, so that the basal lobes do not expand laterally. This gives basal lobes much smaller than in *Phacopidina*. The allied American genus *Scotiella* Delo has small, apparently differently shaped basal lobes, and agrees with *Phacopidina* in more or less obsolescent first and second glabellar furrows. The group of European species centring around forms like *Dalmanitina socialis* (Barrande) as genotype and *D. kegei* R. & E. Richter (1927) have all three pairs of glabellar furrows well developed.

(2) The genal angles are produced into short spines. This does not happen in the genotypes of *Acaste* and *Scotiella*, but it does occur in *Dalmanitina*.

(3) A low, rounded, longitudinal ridge arises beneath the end of the axis of the pygidium and is continuous over the mucro as a rounded thornlike caudal spine, inclined upwards and out of the general plane. In *Acaste* there is no such ridge and in species like the genotype *A. downingiae* (Murchison) no caudal spine. The pygidium of *Dalmanitina*, however, is very like that of *Phacopidina*. *Phacopidina* includes *P. harnagensis* n.sp. (genotype), *P. apiculata* (Salter), *P. major* (Harper), and numerous *P. aff. apiculata*. As Harper says, McCoy's figures of *P. apiculata* exaggerate the first and second glabellar furrows (1851, plate i G, figures 17-18), and the same is true of Salter's drawings of *P. major* (1864, plate i, figure 36). Species left in *Dalmanitina* include *D. socialis* (Barrande), *D. socialis* var. *primaeva* Emmrich (Thadeu, 1947), *D. vetillarti* (Rouault) which may be the same as *D. macrophthalmus* (Brongniart) (cf. Born, 1916), *D. incerta* (Salter), and probably *D. (?) phillipsi* (Barrande) and *D. (?) dujardini* (Rouault). *D. (?) phillipsi* with more parallel-sided glabella and poorly developed first and second glabellar furrows than most Dalmanitinae, and *D. (?) solitaria* (Barrande) come close to *Phacopidina harnagensis* n.g. et n.sp. but the former lacks a caudal spine and the latter has a much larger one than in the Shropshire species. *P. harnagensis* also has angulation of narrow anterior border, a feature in which it compares with *D. robertsi* (Reed) from Haverfordwest.

## GENOTYPE

*Phacopidina harnagensis* n.g. et n.sp.

**Phacopidina harnagensis** n.g. et n.sp.

Figures 24 and 25, plate 10

## LOCALITY AND HORIZON

Side of cartway about 70 yd. north of extreme south end of Smeathen Wood, Horderley, in lower 20 ft. of the Harnage Shale. Bancroft Collection: Horderley W5. *Reuscholithus reuschi* zone.

#### AFFINITIES

This form differs from *Phacopidina apiculata* in its wider glabella and smaller eye. The eye is opposite the faintly defined first and second glabellar lobes, whereas in *P. apiculata* it extends back opposite the front half of the third pair. The pygidium in *P. harnagensis* seems to be shorter with fewer segments. Differences from the Lower Longvillian *P. major* are of a similar nature, but less marked. *P. harnagensis* and *P. major* may be in the same lineage.

The sharp anterior pointing of the basal lobes of the glabella in *P. harnagensis* is naturally most extreme in the internal mould, but may help in distinguishing it from species of *Scotiella*.

#### Family CHEIRURIDAE Salter

#### Genus *Nieszkowskia* Schmidt

#### *Nieszkowskia stubblefieldi* n.sp.

Figures 26 and 27, plate 10

#### DIAGNOSIS

Glabella weakly convex; sides gently curved, converging forward slightly as far as, and more so in front of, second pair of glabellar furrows. Furrows sharply incised, deep, directed obliquely backwards, inner extremities equidistant from median line, or last pair reaching somewhat nearer it; first and second pairs gently convex in front; second pair a little longer than first, third pair similar, but sharply deflected backwards at inner ends, not isolating basal lobes. Surface of glabella and occipital ring uniformly covered with numerous conspicuous tubercles.

Pygidium with three rings on axis (not counting anterior articulating half-ring), and a lozenge-shaped posterior part. The first and third full rings bounded at sides by clearly defined axial furrows; axial furrows absent on intervening segment. Pleural segments produced into two pairs of wide flat spines; the first pair apparently long, tapering, outer sides parallel, each with a slot-like pit diverging from near axial furrow; second pair of spines narrower and shorter than first, sides parallel at first, then meeting at acute angles. Surface of axial lobe covered with numerous large tubercles; a few similar, but widely separated, tubercles on the spines.

#### LOCALITY AND HORIZON

Side of old cartway, about 70 yd. north of extreme south end of Smeathen Wood, Horderley, in lowest 20 ft. of Harnage Shale. Bancroft Collection: Horderley W5. *Reuscholithus reuschi* zone.

#### AFFINITIES

Reed's figures of *Nieszkowskia unica* (Wyville Thomson) (1906, plate xviii, figures 15 and 16) indicate both pleural segments separated by furrows from the pygidial axis, but that species and *N. stubblefieldi* n.sp. may be derived from a common source.

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## EXPLANATION OF PLATES 9 TO 11

[Numbers in square brackets refer to the Register in the Geology Department, British Museum (Natural History), London, S.W. 7. Other figured specimens are thought to be in the Bancroft Collection at the Museum, but have not so far been recognized.]

### PLATE 9

#### *Salterolithus smeathenensis* n.sp.

FIGURE 1. ( $\times 2$ .) Cephalon. Dorsal fringe plate.

FIGURE 2. ( $\times 2$ .) Cephalon. External impression of ventral fringe plate. [In 42082.]

FIGURE 2a. ( $\times 2$ .) Pygidium. Plasticine cast from external impression. [In 42092.] 10 to 20 (?) ft. above base of Harnage Shales, 70 yd. north of south end of Smeathen Wood, Horderley.

#### *Reuscholithus reuschi* Bancroft

FIGURE 3. ( $\times 2$ .) Cephalon. Dorsal fringe plate. Figured by Bancroft, 1929, plate I, figure 3. [In 42080.] Harnage Shales, Smeathen Wood, Horderley.

#### *Broeggerolithus broeggeri* (Bancroft)

FIGURE 4. ( $\times 2$ .) Cephalon. Dorsal fringe plate. Figured Bancroft, 1929, plate i, figure 6.

FIGURE 5. ( $\times 2$ .) Pygidium. Figured Bancroft, 1929, plate i, figure 7. Glenburrell beds (Lower Soudleyan), from Stream Section at north-east corner of Smeathen Wood (Bancroft Collection: Horderley P 10.)

#### *Broeggerolithus soudleyensis* (Bancroft)

FIGURE 6. ( $\times 4$ .) Anterior part of cephalon with 8 large pits of  $E_1$  external. Pale sandstones of *B. soudleyensis* zone, from quarry east of Glenburrell Farmhouse, Horderley.

#### *Marrolithus ultimus* n.sp.

FIGURE 7. ( $\times 2$ .) Cheek and dorsal fringe plate. *Horderleyella plicata* zone, Hoar Edge Grit (Costonian) in quarry, 500 ft. south of Coston Farmhouse, south of Aston-on-Clun.

#### *Broeggerolithus transiens* (Bancroft)

FIGURE 8. ( $\times 2$ .) Cephalon. Dorsal fringe plate. Figured Bancroft, 1929, plate ii, figure 5. [In 42071.] *Wattsella unguis* Beds (Marshbrookian), 15 ft. above top of *W. watsi* beds; top of Old Quarry face, in Marsh Wood, Marshbrook.

#### *Onnia gracilis* (Bancroft)

FIGURE 9. ( $\times 2$ .) Cephalon. Dorsal fringe plate. Figured Bancroft, 1929, plate ii, figure 8. [In 42074.] Onnian, 40 yd. west of 'Cliff' section, Onny River, near Wistanstow.

#### *Onnia cobboldi* (Bancroft)

FIGURE 10. ( $\times 2$ .) Cephalon with dorsal fringe plate seen from side.

FIGURE 11. ( $\times 2$ .) Cephalon. Dorsal fringe plate. Figured Bancroft, 1929, plate ii, figure 6. [In 42072.] Onnian, about 90 yd. west of 'Cliff' section, in north bank of Onny River.

*Onnia superba* (Bancroft)

FIGURE 12. ( $\times 2$ .) Cephalon. Dorsal fringe plate. [In 42107.] Onnian, east part of 'Cliff' section, '21 ft. below base of Silurian', north bank of Onny River. (Bancroft Collection: P 3.)

*Onnia superba* n.var. ?

FIGURE 13. ( $\times 2$ .) Cephalon. Dorsal fringe plate. Same locality as for figure 12.

*Salterolithus* (*Ulricholithus*) *ulrichi* n.subgen. et n.sp.

FIGURE 14. ( $\times 2$ .) Cephalon. Dorsal fringe plate. [External impression, in 42371.] *Ulricholithus ulrichi* zone, in stream section, about 120 yd. west-north-west of Middle House, north of Welshpool, Montgomeryshire.

## PLATE 10

*Salterolithus* aff. *harnagensis* Bancroft

FIGURE 15. ( $\times 2$ .) Cephalon. Decorticated dorsal fringe plate. [In 42093.] Fissure in Uriconian, quarry east of Church Stretton.

*Onnia superba* (Bancroft)

FIGURE 16. ( $\times 2$ .) Cephalon. External impression of ventral fringe plate. [In 42094.] Highest zone of Onnian, 'Cliff' section, north bank of Onny River, near Wistanstow.

*Lonchodomas pennatus* (La Touche)

FIGURE 17. ( $\times 2$ .) Partly decorticated cephalon. [In 42096.] *Onnia gracilis* zone, in river-bed, 40 yd. west of 'Cliff' section, Onny River.

*Raphiophorus edgelli* Cowper Reed

FIGURE 18. ( $\times 2$ .) Pygidium. [In 42105.] *Reuschella semiglobata* beds (Actonian), Jack Slither, Onny River.

*Remopleurides burmeisteri* n.sp.

FIGURE 19. ( $\times 2$ .) Cranidium. Natural mould. [In 42106.]

FIGURE 20. ( $\times 2$ .) Free-cheek. Ditto. [In 42098.] Both from the *Onnia superba* zone, 'Cliff' section, Onny River.

*Acidaspis harnagensis* n.sp.

FIGURE 21. ( $\times 4$ .) Cranidium. Natural mould. [In 42086.]

FIGURE 22. ( $\times 2$ .) Pygidium. [In 42087.] ? 10 to 20 ft. above base of Harnage Shales, 70 yd. north of south end of Smeathen Wood, Horderley.

*Proetidella fearnsidesi* n.g. et n.sp.

FIGURE 23. ( $\times 2$ .) Dorsal shield (external impression). [In 42083.] Same locality as for figures 21 and 22.

*Phacopidina harnagensis* n.g. et n.sp.

FIGURE 24. ( $\times 2$ .) Decorticated cephalon with 7 thoracic segments. [In 42087.]

FIGURE 25. ( $\times 2$ .) Pygidium. [In 42088.] Same locality as for figures 21 and 22.

*Nieszkowskia stubblefieldi* n.sp.

FIGURE 26. ( $\times 2$ .) Glabella. Natural mould. [In 42084.]

FIGURE 27. ( $\times 2$ .) Pygidium. Ditto. [In 42085.] Same locality as for figures 21 and 22.

## PLATE 11

*Diacalymene praecox* n.sp.

FIGURE 28. ( $\times 2$ .) Cranidium. Internal mould. [In 42090.]

FIGURE 28a. ( $\times 2$ .) Profile. ? 10 to 20 ft. above base of Harnage Shales, Smeathen Wood, Horderley.



*Flexicalymene acantha* n.sp.

FIGURE 29. ( $\times 2$ .) Cranidium. Internal mould. [Holotype, In 42091.]

FIGURE 30. ( $\times 2$ .) Pygidium. Ditto. Probably belonging to this species. Same locality as for figures 28 and 28a.

*Flexicalymene trigonoceps* n.sp.

FIGURE 31. ( $\times 2$ .) Cranidium. Internal mould. [Paratype, In 42104.] *Wattsella unguis* beds, Upper Longville Flags (Marshbrookian) in Old Quarry, Marsh Wood. Bancroft Collection: Horderley Z1.

FIGURE 32. ( $\times 2$ .) Cranidium, thorax, and pygidium. Internal moulds. [Holotype, In 42102.] *W. unguis* beds, Lane section, Marsh Wood, Marshbrook. Bancroft Collection: Horderley Z3.

FIGURE 32a. Profile of cranidium. Same locality as for figure 32. During reproduction this profile has been wrongly inserted with the anterior margin pointing towards bottom of the plate.

FIGURE 32b. Reconstruction of pygidium, based on material from same locality as for figure 31.

*Flexicalymene laticeps* n.sp.

FIGURE 33. ( $\times 2$ .) Cranidium. Internal mould. [Holotype, In 42103.]

FIGURE 33a. ( $\times 2$ .) Profile of cephalon. Acton Scott beds (Actonian), in wood, west of Rose Villa, Marshbrook: *Reuschella semiglobata* fauna.

*Flexicalymene salteri* n.sp.

FIGURE 34. ( $\times 2$ .) Cranidium. [Paratype, In 42099.] Acton Scott beds with *R. semiglobata*, Jack Slither, Onny River.

FIGURE 34a. ( $\times 2$ .) Profile. Same locality as figure 34.

FIGURE 35. ( $\times 2$ .) Glabella. Internal mould. [Holotype, In 42100.] Acton Scott beds with *R. semiglobata*, in wood, west of Rose Villa, Marshbrook.

*Flexicalymene onniensis* Shirley

FIGURE 36. ( $\times 2$ .) Pygidium. [In 42095.]

FIGURE 37. ( $\times 2$ .) Cranidium. [In 42097.] Acton Scott beds (Onnian), about 40 yd. west of the 'Cliff' section, Onny River, near Wistanstow, *Onnia gracilis* zone.

*Broeggerolithus constrictus* n.sp.

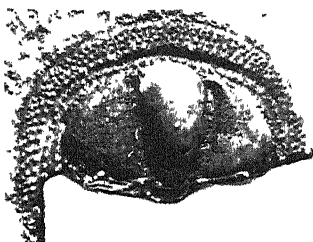
FIGURE 38. ( $\times 3.5$ .) Cephalon, partly decorticated, with dorsal fringe plate showing sudden termination of  $E_2$  row of pits laterally and associated constriction of outline. [Holotype, In 42101.] *B. constrictus* beds (Basal Soudleyan). South side of stream from Ceunant to below Cockshut, near Welshpool, Montgomeryshire.

The photographs reproduced in the plates are the work of H. Britten.





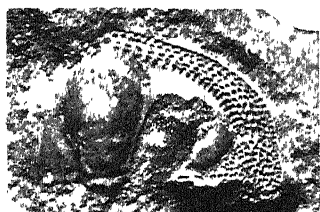
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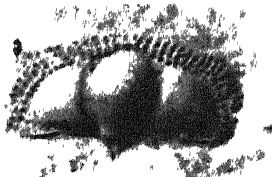
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2a



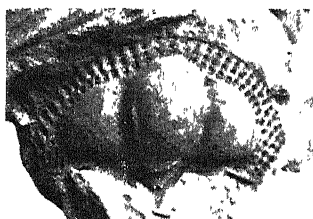
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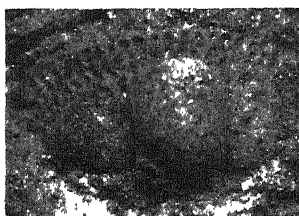
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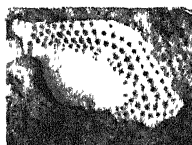
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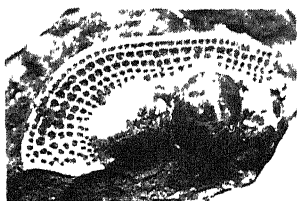
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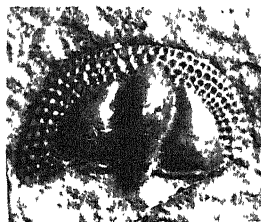
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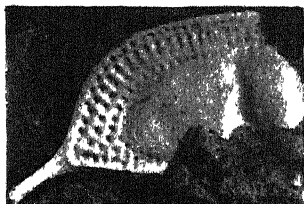
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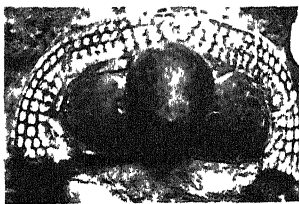
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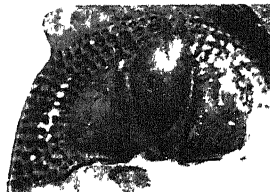
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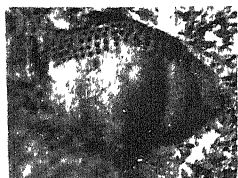
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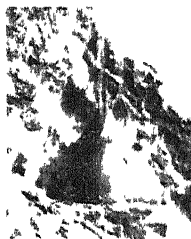
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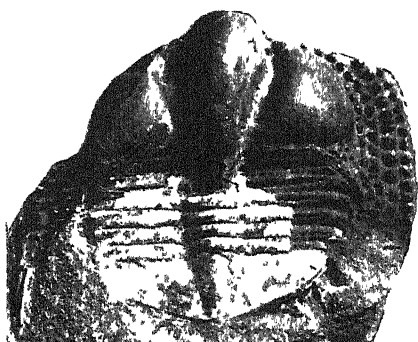
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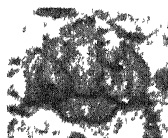
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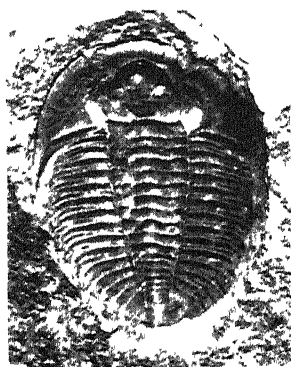
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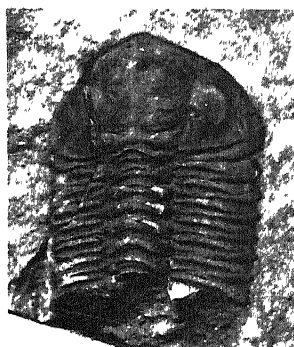
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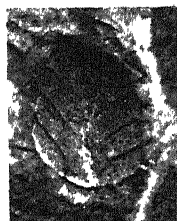
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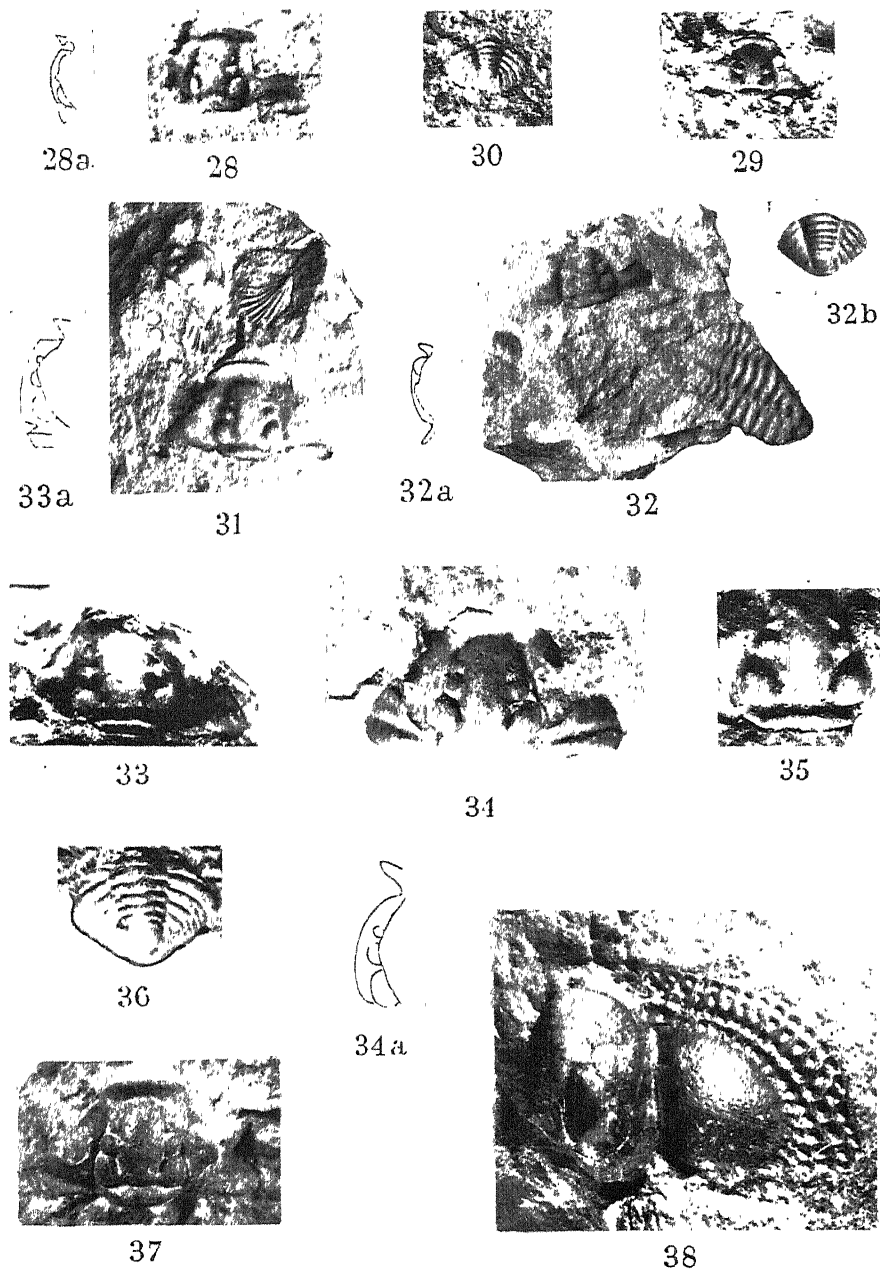
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# The presentation of scientific information

BY E. N. DA C. ANDRADE, F.R.S.

*(Lecture delivered before the Royal Society Scientific Information Conference  
on 23 June 1948)*

[Plate 12]

When the organizers of this conference did me the honour to suggest that I should give this lecture on some general aspect of the theme that is engaging our deliberations, I was somewhat at a loss to know what plan to adopt. Like most of those present I have, in one way and another and at various times, been engaged in the communication of scientific information, but that does not necessarily make it an easy subject to discuss. Not to weary you with my doubts and inward debates, I finally decided to attempt a brief historical survey of the subject, for to see how one's difficulties arose is sometimes a step towards their solution. In any case I hope that the considerations that I shall lay before you may prove a mild and gentle, if not soporific, interlude in the strenuous deliberations upon which you are engaged.

I wish, then, to consider how the communication of scientific information—more particularly the communication of original scientific discovery, of even the smallest degree of importance and interest—has grown to be the very complex matter that it is to-day. The methods of communication that we shall have to consider are, I suppose, the spoken word, the book and the scientific periodical, which to-day includes both the periodical publishing original papers and the periodical publishing abstracts of papers. You may be surprised that I have included the spoken word, or, if you prefer it, personal communication, but I think that, if you consider, you will come to the conclusion that this personal method still retains its importance. I do not include radio under this head, because we are, I think, concerned rather with a first record of novel scientific results than with the popular exposition of general scientific advances. I would remind you that even in our times when students from all over the world flock to such a laboratory as Rutherford's (I choose a man no longer with us to avoid a selection among the living that might be invidious) it is not so much to avail themselves of material instruments—the instruments used by Rutherford could be bought by laboratories in any country—nor to hear a statement of what the leader has already achieved, which can be read in the scientific journals, as to hear from the leader's mouth what is going on in his head at the moment, to learn of scientific progress in the making. The writing of a paper comes, in general, after the results have already been communicated to a small circle in the laboratory and their friends. The importance of the spoken word has not yet vanished, and it would do no harm if some of our great men, whose audiences are attracted by their fame and retained in their seats by considerations of courtesy, were to remember that he who uses the spoken word should pay some attention to acquiring proficiency in the use of the instrument, just as he takes trouble in learning to handle any other

instrument of his profession. Occasionally the performance brings to mind the man who was asked if he could play the violin and replied that he did not know—he had never tried. The ability to present scientific facts clearly in a lecture is not a gift from heaven; I doubt very much if anybody gives a really good first lecture. At any rate, taking one of the best British lecturers of modern times, one who was particularly good at communicating new scientific advances, Sir William Bragg, it is recorded of him that in his early days as professor he was one of the least impressive lecturers, but by careful application he attained that high eminence in the art for which he was later known.

Let us now look back to the beginnings of science and consider—very briefly—the communication of knowledge in the early days of Greek science. The spoken word at that time was supreme, although, of course, the written scroll transmitted the teaching. It would weary you were I to attempt to picture the great schools of antiquity—Plato's Academy, the Schools of Athens, of Pergamon, of Crotona are familiar to all of you. What we are apt to forget, however, is the freedom with which men travelled great distances in those days in order to learn by the spoken word of the advances in science and in philosophy. Plato's Academy was at Athens, but he thought nothing of visiting Sicily, and the students and philosophers of classical times travelled from Greece to Asia Minor, the Black Sea, Alexandria, Italy and North Africa as a matter of course. Since the days of the pre-eminence of Athens there has probably never been a time when personal intercourse among the learned of Europe was more set about with difficulties than it is to-day, which enhances for us the importance of the written word.

I would not, of course, wish to minimize the part played by the manuscript in early science. The great historian Mommsen, for instance, says, writing of the times of Julius Caesar: 'Reading had become a fashion, nay a mania... The superior officer was seen in the camp-tent with the obscene Greek romance, the statesman in the senate with the philosophic treatise in his hands.' The two libraries of Alexandria, finally destroyed by the Saracens, must, in their prime, have contained at least half a million books, of which a fair proportion dealt with the science of the ancient world, and there were considerable libraries at Rome, Athens, Ephesus, Timgad, Constantinople and other centres of culture. Mention of Alexandria calls to mind that the Greeks had no sacred books, which was at that time advantageous for scientific advance, as illustrated by the traditional reply of the Caliph to the general who wished to spare the Alexandrian books from destruction—'if these writings of the Greeks agree with the Book of God, they are useless and need not be preserved: if they disagree they are pernicious, and ought to be destroyed.' The word of the teacher was transmitted by document—we have Tennyson's picture of Lucretius, passing

To turn and ponder those three hundred scrolls  
Left by the Teacher, whom he held divine

—a considerable library. We have, then, in classical times, the great teacher, attracting scholars from all over the learned world, and the manuscript document as the two important methods of scientific communication. There were, needless to say, no scientific periodicals.



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Turning to the Middle Ages and the revival of learning, we have the same methods for the communication of scientific ideas, the manuscript and the spoken word, with the same very great importance attached to the spoken word, partly on account of the cost of books and partly on account of the enthusiasm which brought students from all over Europe to centres of learning. In the fifteenth century there were some sixty universities in Europe; even when it is admitted that some of them were of very minor importance the number is impressive. The travels undertaken by learned men, no matter what their particular interests, and the international character of learning in what are called 'the Dark Ages', were extraordinary. Relations among men of science of all countries were, of course, much facilitated by the existence of what we no longer have, an international language of learning. Latin was the medium of expression in university and medical school, practically all books were written in Latin, and it must be remembered that it was not until round about 1700 that the man of science regularly expressed himself in his native language. Newton's *Principia* (1687) was written in Latin, his *Opticks* (1704) in English, and while, of course, I know that many scientific books were written in English, French and Italian before 1700, and many in Latin after 1700, yet it is a convenient approximate date to take for the transition.

And here, perhaps, I may interpolate a word as to the language most used in science to-day. In 1934 Sir Charles Sherrington made an examination of the *World List of Scientific Periodicals* and counted the number of scientific periodicals published in five great languages. The result was:

Russian	1,833	scientific periodicals	
Italian	1,667	"	"
German	6,186	"	"
French	5,013	"	"
English	13,494	"	"

The total number for the other four languages does not much exceed the number for English. It is quite clear, then, that if there is an international language in science to-day it is English. I take pleasure in quoting the last sentence of Sherrington's letter,\* which runs as follows: 'The results cited above make evident the special responsibility resting on those contributors to science whose language is English to spare no pains to write it worthily of the great role entrusted to it as a medium of scientific thought to-day.'

Let me return to the Middle Ages and consider a few examples of the international character of learning. Saint Thomas Aquinas was born near Naples round about 1225 and, after a preliminary education at Monte Cassino and Naples, later studied in Paris for three years and then went to Cologne. He returned to Paris to teach there, and later taught at Rome, Paris again and Naples. He died on his way to Lyons. You will say that he was not a scientist, but he took an interest in the science of his times and was a very great scholar. Perhaps you prefer to consider Roger Bacon, who was born in Somerset and studied at Oxford (though not at Brasenose,

\* *Nature*, 134, 624 (1934).

as tradition asserts, for it was not founded until after his death). He taught for years at Paris, which was the centre of the intellectual life of the Middle Ages. Perhaps a later name, a forerunner of modern science, will appeal more, Vesalius, a Fleming, who studied at Louvain, Montpellier and Paris, and then went to Italy. There in 1537, at the age of twenty-two, he was appointed professor at Padua, where he spent seven years. Later he went to Basle, where he lectured while his great book, *De Humani Corporis Fabrica*, was passing through the press there. After that he went to Spain, Cyprus, Jerusalem, and died on his way to Padua, whither he had been summoned to succeed Fallopius. Later, our John Dee, who was born in 1527 and wrote a very remarkable preface to the first English translation of Euclid, as well as valuable books on navigation, travelled as a young man in the Low Countries, forming friendships with many mathematicians of fame, studied at Louvain, a great centre of learning where van Helmont was later, and went to Paris, where he lectured to audiences so large that the hall would not hold them, with the result that many of the students clambered up outside the windows, so that they could see him even if they could not hear him. He corresponded with men in the Universities of Orleans, Cologne, Heidelberg, Strasbourg, Verona, Padua, Ferrara, Bologna, Urbino and Rome. His subsequent travels took him to Antwerp and the Low Countries again, Bremen, Lubeck, Cracow, Prague and Central Europe in general. Cardan, the celebrated Italian doctor and mathematician, travelled over the whole of Italy and in Germany, Switzerland, the Tyrol, France, England, Scotland and the Low Countries. Certainly personal contacts and private correspondence were among the main means of communicating knowledge in those times.

But I must not weary you, who are filled with the glory of the present and the faith of the future, with further stories of the remote past. The coming of printing lent a much increased importance to the written word as a means of communication. It made the words of the Arabian and classical writers on science widely accessible—and it must be remembered that medieval medicine was dominated by the Arabs. The first Euclid was printed in 1482 at Venice; the works of Pliny, Hippocrates, Galen, Aristotle, Celsus and Dioscorides were printed frequently in the period between 1469 and the end of the century.

The first great scientific revelations made in print were in the *De Revolutionibus* of Copernicus in 1543, and in Vesalius's *De Humani Corporis Fabrica* which appeared in the same year; the seventeenth century opened with Gilbert's *De Magnete*; Harvey's *De Motu Cordis* appeared in 1627; Kepler's *Astronomia Nova* and *Harmonices Mundi* in 1609 and 1619; Galileo's *Dialogo* in 1632. By 1660, the year when the Royal Society was founded, the printed book was thoroughly established as a means of communication, although the private letter was still favoured—see, for instance, the *Commercium Epistolicum* printed at Oxford in 1658 in which letters on mathematics exchanged between Brouncker, Kenelm Digby, Fermat, Frenicle, Wallis and Schooten are printed. As examples of earlier writers of letters that conveyed and discussed the scientific news of the day may be mentioned Descartes and Mersenne—the latter using 'barbarous Latin and pitiful French', according to Duhem.

The foundation of the Royal Society marks the beginning of a new stage in scientific communication, the scientific periodical, as well as the inception of the modern scientific society. Actually the Accademia del Cimento was founded earlier, in 1657, and published in 1666 an account of its experiments in a book which exercised a great influence in its time, the *Saggi di Naturali Esperienze*, which, appearing in Italian, the vernacular (an unusual thing), was translated into Latin and English, possibly into other languages. The Accademia lived, however, for only ten years. The Accademia dei Lincei, which lasted from 1600 to 1630, was even earlier, and had the support of Galileo. But of the scientific societies which have endured the Royal Society was the first, and its *Transactions*, which have now been a channel of scientific communication for 283 years, were first published in 1665. In the beginning these *Transactions* were published, not by the Society, but by the Secretary on his own account, and contained descriptions not only of what was communicated to the Society by Fellows, but also of work done abroad—thus in the very first number there is ‘An Accompt of the Improvement of Optick Glasses at Rome’, ‘A Catalogue of the Philosophical Books publisht by Monsieur de Fermat at Tholouse, lately dead’, and so on. The *Transactions* had, in fact, something of an international character, for foreigners sometimes carried on scientific controversy in its pages. When the *Transactions* temporarily ceased publication in 1676 Robert Hooke personally undertook the publication called *Philosophical Collections*, which went on until 1682. These *Collections* contain, besides communications from Fellows, accounts of matters of scientific interest abstracted from foreign journals or books, such as the description of Lana’s flying chariot taken from his *Prodomo*, with a criticism by Hooke, and the review of Borelli’s *De Motu Animalium*. In 1683 the *Transactions* appeared once again, and since that time have continued without interruption. The scientific journal was thus established.

Actually the first number of the *Journal des Sçavans*, a privately published periodical, appeared a few months before the first number of the *Transactions*. The *Journal* published accounts of scientific discussions; reviews of books, classical, literary and theological as well as scientific; and notices of men of science newly dead, such as the notice of Fermat which appeared in an early number. The *Journal* was, perhaps, more closely akin to *Nature* than to the *Transactions*. It must have been popular, for it appeared in two formats, as a folio\* at Paris and as a 12mo in Amsterdam. It is amusing to note, as showing how little reliance can be placed on some learned authorities, that Martha Ornstein† quotes Zedler’s ‘fluchtigblutige Franzosen haben Ekel vor Folianten’ (‘volatile Frenchmen dislike folios’) to explain why the *Journal des Sçavans* was so small and the *Acta Eruditorum* (‘huge folios’ according to her, but in fact ordinary quartos) so large. Actually the standard edition of the *Journal* is slightly larger than the *Acta*.

The *Acta Eruditorum* first came out in 1682 and was published in Latin at Leipzig, with the support of the Elector of Saxony. A large part of it is taken up with book reviews, not only of scientific, but also of theological, philosophical, legal and other books. It also contains, however, original contributions by outstanding

\* While having the usual form of a quarto (page  $8\frac{3}{4} \times 6\frac{3}{4}$  in.) the book collates as a folio.

† *The Role of Scientific Societies in the Seventeenth Century*, 1928.

men of science. The volume for 1688, for instance, includes a 7-page review of Stillingfleet's *The Antiquities of the British Churches*, a 6-page review of Boyle's *Disquisition about Final Causes* and a 12-page review of Newton's *Principia*, and Latin translations of papers that had appeared in our *Philosophical Transactions*, as well as original papers by Papin, and Jacob Bernoulli. The *Acta* thus gave a general account of what was going on in the world of learning. Another German scientific journal which appeared about this time was the *Miscellanea naturae curiosorum*, while the famous Thomas Bartholinus brought out in Copenhagen the *Acta medica et philosophica hafniensia*, which lasted from 1673 to 1680. The *Nouvelles de la republique des lettres* appeared in 1684, in Holland, and gave accounts of affairs in the scientific world. It did not last for long, but had imitators. Thus by the end of the seventeenth century scientific journals, both those designed for the publication of original work and those of a more popular nature, had made their appearance and established their places as organs of communication between men of science.

Perhaps I may here refer to a curious undertaking of which I can find no mention except a mere citation in certain catalogues, the *Weekly Memorials for the Ingenious*. These 'Memorials' were weekly collections of reviews of current books, mostly of scientific interest 'with some other curious *Novelties* relating to *Arts and Sciences*'. Fifty parts appeared for the year 1682, and were afterwards bound in one volume.\* In the preface the author (— Beaumont, according to the only authority† that mentions him) refers to Oldenburg and his *Philosophical Transactions* and to Hooke and his *Philosophical Collections* and says of the Royal Society: 'If the R.S. shall think my endeavours in this kind any way subservient to their *designes*, it may animate my *industry* to perform things in the best manner I may, none being more devotedly their Servant than my self.' Among the authors of whose books account is given are Caspar Bartholinus, Borelli, Jacob Bernoulli, Boyle, Fermat, Malpighi and Papin. Many of the reviews are taken from the *Journal des Sçavans*, a few from the *Acta Eruditorum*. Such a weekly review of scientific books has no counterpart, I believe, to-day.

Before leaving the period I should, perhaps, mention the works of an encyclopaedic character that became popular in the sixteenth and seventeenth centuries, and did much to spread knowledge. Conrad Gesner, of Zürich, was a naturalist who brought out a great *Historia Animalium* in four volumes (1551–58), from which our Topsel compiled his *Histories of Foure-Footed Beastes* (1607). Aldrovandus brought out a great book on birds, Moufet on insects. Everyone knows of Gerarde's *Herball* (1597), founded on the earlier work of Dodoens, and typical of a number of herbals which have been fully described by Agnes Arber.‡ All these books were beautifully illustrated. Reference may also be made to the appearance of German works which gave a general account of all that was known in wide fields of physical science at the time, including the most recent advances—forerunners, in a way, of the modern

\* The history of this periodical is somewhat complicated. There were two completely different and rival issues for a certain period.

† Halkett and Laing, *Dictionary of Anonymus and Pseudonymous English Authors*.

‡ *Herbals: Their Origin and Evolution*, 1912; 2nd ed. 1938. Cambridge University Press.

German 'Handbuch'. Such works were Schwenter's *Deliciae Physico-Mathematicae* (1651) in three parts of some 600 pages each; Gaspar Schott's *Magia Universalis* (1658) in four volumes, and his *Mechanica Hydraulicopneumatica* (1657), in which the first account of Guericke's pump was given;\* and Sturm's *Collegium Experimentale* (1676). All these works were intended for a wide circle of readers, and if, in general, they contained little in the way of first statement of original discoveries, did much to spread scientific knowledge.

At the opening of the eighteenth century we find a publication answering a new need. *Miscellanea Curiosa* in three volumes (1705-1707) contained a selection of the most important papers which 'being register'd in the Voluminous Journals of the *Royal Society* are amongst a multitude of less useful Matters, so Obscurely hid, that but very few inquisitive Gentlemen ever so much as heard of them'. The names of Halley, Newton, Clopton Havers, John Wallis and de Moivre occur freely as authors, Halley being particularly well represented. The papers are not abridged, for, as the preface says, they are in many cases already abridgments themselves. What we have here, then, is in some way a forerunner of the 'Progress Reports', which are an essential feature of our modern machinery of information. We also find, in 1704, the issue of the first technical dictionary in any language, the *Lexicon Technicum* of John Harris, of which a second volume appeared in 1710, notable for containing the first appearance of Isaac Newton's note *De Natura Acidorum*, to the importance of which Academician Sergei Vavilov recently directed attention.† The *Lexicon Technicum*, which was in English, contained articles on, for instance, Newcomen's newly invented steam-engine, and on the microscope, illustrated with a plate of John Marshall's 'new invented double microscope', and went into several editions (5th ed. 1736). We can scarcely mention encyclopaedias as a medium for the communication of scientific knowledge without passing reference to the great French *Encyclopédie* of Diderot, which was originally to have been a French translation of Ephraim Chambers's *Cyclopaedia*, but developed into a very much larger work, a vast encyclopaedia of science and, in particular, applied science and manufacture. D'Alembert was responsible for the mathematical articles, and Voltaire was among the contributors. The first volume appeared in 1751, the last volume of the letterpress in 1765 and the final volume of the plates in 1772. It caused much political controversy: why, it is hard to understand nowadays.

The first half of the eighteenth century saw the foundation of the Academy of Sciences of St Petersburg in 1725, now the Academy of Sciences of the U.S.S.R. Just after the end of the war the 220th anniversary of its foundation was made the occasion of a magnificent celebration, which some of us had the good fortune to attend. The first volume of its *Mémoires* appeared in 1728. Not long after this the *Proceedings of the Royal Swedish Academy* began (1739), to be followed in a few years by the *Proceedings of the Royal Danish Academy* (1745). At that time, of course, Germany was a body of independent States of which Hanover was the first to produce a scientific journal in the *Verhandlungen der Königlichen Akademie der*

\* Guericke's own description first appeared in his *Experimenta nova, ut vocant, Magdeburgica de vacuo spatio*, in 1672.

† *Royal Society Newton Tercentenary Celebrations*, 1947.

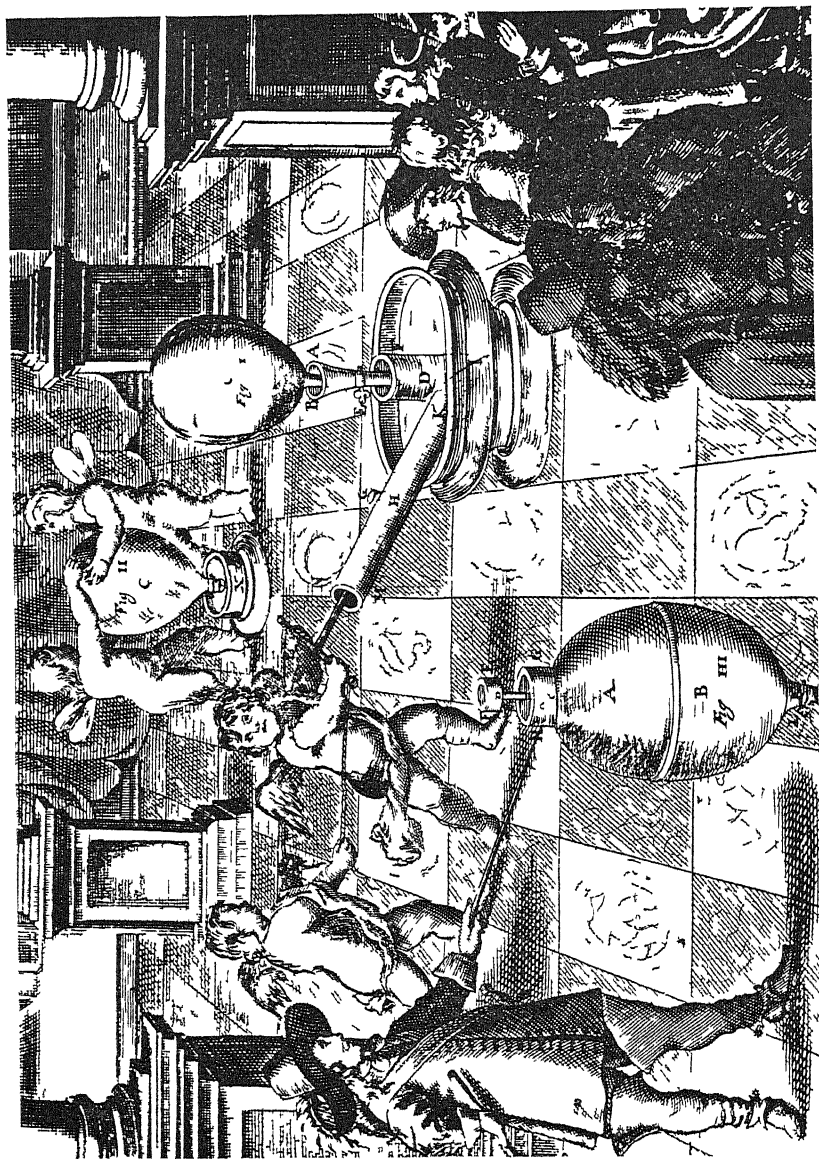


FIGURE 1 The first representation of Gueucle's air pump, from Gaspar Schott's  
*Mechanica Hydraulico-pneumatica*, 1657

*Wissenschaften zu Göttingen* in 1752. As the University there was founded by our George II in 1734—his bust dominates the Aula—we may take a particular interest in this publication. I mention the appearance of these four journals to remind you that, although the first half of the eighteenth century was not remarkable for great scientific discoveries, yet it saw a great activity in the production of media for their publication.

Through the eighteenth century there continued the lecture, the book and the scientific journal as means of communication. Lectures for interested subscribers became a feature of the cultured life of London: for instance, in 1717, Desaguliers published what he calls in the preface 'Minutes of my lectures, for the Use of such Gentlemen as have been my Auditors'; another book of about this date describes a course of experiments to be performed by Francis Hauksbee, with explanatory lectures read by William Whiston.

The end of the century saw a fresh great development of the channels of scientific information in the periodical limited to a particular branch, or to certain closely allied branches, of science. In 1771 came forth the *Transactions of the American Philosophical Society*, with which event Benjamin Franklin was prominently associated. This publication has continued without a break to the present day. The *Annales de Chimie et de Physique* first appeared in 1789 and was followed by the *Annalen der Physik und der Chemie* (which developed out of the *Journal der Physik*, started in 1790), both of which exist to this day, except that the chemical part of the title has disappeared. Our own *Philosophical Magazine* first came out in 1798. Thus by the end of the century England, France and Germany all possessed journals for the publication of original work in physics, which have continued to the present day.

Another event of importance was the establishment of the provincial scientific society. I do not pretend to have entered upon a careful investigation as to which was the first, for it would be a long and argumentative undertaking. In any case, typical of what I mean is the Manchester Literary and Philosophical Society, whose *Memoirs* came forth in 1789. John Dalton joined the Society in 1794, and became President in 1817, an office which he continued to hold until his death in 1844. Many of his papers, on a variety of subjects, appeared in the *Memoirs* of the Society. In the early years of the present century Rutherford and his collaborators contributed papers and the Society still maintains its high reputation. The Manchester Literary and Philosophical Society is an outstanding example, but in general the local scientific societies fulfilled an important function in the spreading of scientific information, especially in the field of natural history. In those times the country parson was often a well-trained naturalist, sometimes an astronomer, and he did much to foster, through the local society, a taste for science and, occasionally active scientific work. To-day, in general, he has neither the training, the background nor the leisure necessary for such work, and, whether due to this or to other causes, the local societies and clubs seem, unfortunately, to be diminishing in number and activity, although some preserve their status.

The early years of the nineteenth century saw the first appearance of scientific journals in the modern sense in certain countries justly celebrated for the brilliance

of their scientific achievements. In 1812 were born the *Proceedings of the Royal Academy of Sciences of Amsterdam*; in 1819 the *Memoirs of the Royal Lombard Institute of Science and Letters* announced the growing activity in Italy. Belgium followed in 1835 with the *Bulletin de l'Académie Royale des Sciences et Belles Lettres de Bruxelles*. In 1842 came forth the *Acta* of the Finnish Society of Sciences. America already had the *Transactions of the American Philosophical Society*, to which reference has been made, but an important event in her scientific history was the initiation of *The American Journal of Science* (1819), often called *Silliman's Journal* since it was founded by the well-known Benjamin Silliman and carried on by his son, and later came the *Proceedings of the American Philosophical Society* (1840), closely followed by the *Proceedings of the American Academy of Arts and Sciences* (1848).

With the nineteenth century the specialized journal, devoted to one branch, often a very limited branch, of science, began its great development, which, I suppose, is the main cause of this conference. To take a few examples, *Crelles Journal*, where so many great mathematical papers are to be found, first appeared in 1826 and *Dinglers Polytechnisches Journal* in 1820. The flood of specialized chemical journals was heralded by Liebig's *Annalen der Pharmazie* (1832), which in 1840 became the *Annalen der Chemie und Pharmazie*. The chemical encyclopaedias, which culminated in the great Beilstein, which surely must come in for discussion at the conference, began, as far as I know, with the *Handwörterbuch der Reinen und Angewandten Chemie*, which likewise owes its inception to Liebig. It would have been extremely laborious for me to amass, and tedious for you to hear, detailed statistics about the journals of the nineteenth century, but, taking as an example those dealing with physics in all its branches—those from which the data in the physical tables of Landolt-Börnstein are compiled—in the period 1810 to 1910 about eighty new journals appeared, roughly at the rate of eight new journals every ten years. This is only one branch of science, and only up to 1910. You will, no doubt, hear just what the position is nowadays from those more familiar with the situation than I am.

The foundation of the *Versammlungen Deutscher Naturforscher und Ärzte* in 1822 represents a new channel of scientific communication, both by personal contacts and publication—especially the former—which quickly found imitators. It organized annual meetings of men of science in different German towns for the purpose of discussing the latest advances and also of arousing interest in science in the locality. Our *British Association* followed in 1831, and other countries have similar institutions, such as the *Association Française pour l'Avancement de la Science*.

With the enormous growth of science in the nineteenth century a new portent appeared, the abstracting journal. The Royal Society was early in the field, for the *Proceedings*, which appeared in 1832, were in the first case merely a collection of abstracts. It was not until the seventh volume, of 1856, that short original papers began to appear. The abstracts were not of papers in general, or even of all English papers, but only of those that had appeared in the *Transactions* of the Society. Chemical abstracts first appeared in Germany as the *Pharmaceutisches Central-Blatt* in 1830 and in England in the publications of the Chemical Society in 1841, although they were not published separately until 1878. The *Beiblätter* to the *Annalen der*



*Physik* began in 1877 the publication of abstracts of all physical papers except those printed in the *Annalen* itself. About the same time appeared the botanical abstracts *Botanischer Jahresbericht* (1874) and the zoological *Zoologischer Jahresbericht* (1880). By the end of the century many other series of abstracts had appeared, including our *Science Abstracts*, and you know, or will soon know, what the situation is like to-day.

Brief mention must also be made of special bibliographies, such as *The Royal Society Catalogue of Scientific Papers*, which covers the whole of the nineteenth century, and, more particularly, of the periodical bibliographies that, especially in the biological sciences, are widely used to-day. Typical are the *Zoological Record*, which began in 1864, the *Index Medicus* (1879) and the *Index Kewensis* (1893).

I cannot leave the nineteenth century without referring to a development in which England led the world, namely, the appearance of the periodical *Nature* in 1869. The objects were announced to be 'First, to place before the general public the grand results of scientific work and scientific discovery; and to urge the claims of science to a more general recognition in education and in daily life; and, secondly, to aid scientific men themselves, by giving early information of all advances made in any branch of natural knowledge throughout the world, and by affording them an opportunity of discussing the various scientific questions which may arise from time to time'. It would be superfluous here to describe the great part which *Nature* has played and now plays in the communication of scientific information. It is, for many purposes, an international organ which is unique. Where the vexed question of preserving priority arises, a letter to *Nature* is everywhere the solution. In the seventeenth century it was the custom to publish, in the interest of priority, an anagram in which the letters of a significant sentence were arranged in alphabetical order. The best-known example is probably Hooke's *ceiinossttuv* which, properly arranged, makes *ut tensio sic vis*, Hooke's law. The anagram was, of course, unintelligible until the author was challenged by event and construed its meaning. Some writers of letters to *Nature* have a strong historical sense.

It would not be right to omit the book as a channel of original scientific information and a valuable aid and incitement to original research. I have already referred to important early books, such as Copernicus's *De Revolutionibus* and Gilbert's *De Magnete*. These books were widely read—Copernicus, for instance, was printed four times within the hundred years that started with its first appearance, and Gilbert four times within forty years. The influence of Newton's *Principia*, of which three editions appeared in forty years, and of his *Opticks*, of which four editions appeared within thirty years, it would be hard to overrate. I have already noted that in the seventeen years which elapsed between the books Newton had abandoned Latin for first publication and passed to the vernacular, although the *Opticks* was speedily translated into Latin for the benefit of the Continent. He was not, of course, by any means the first to publish in English: Robert Boyle's books all appeared first in English and then in Latin, and there are important Elizabethan scientific books in English. Huygens published his early books, such as the *Horologium Oscillatorium* (1673), in Latin, but the *Traité de la Lumière* appeared in 1690 in French. Thus the turn of the seventeenth century heralded the passing of Latin as a universal

language, although for the next fifty years it is common to find books in that tongue, for instance, those of the Bernoullis and the Eulers. With the general decline of the classical influence dwindled also the use of the dialogue form, no doubt a memory of Plato. Examples of the use of this form are Fernel's *De Abditis Rerum Causis* and Galileo's *Dialogo*, in which there debate Salviati, a Copernican; Sagredo, a witty and educated scholar, desirous to learn; and Simplicio, an Aristotelian who puts forward the stock arguments of the scholiasts in an absurd way. You know, of course, the tradition that Galileo put certain arguments which the Pope had suggested to him into Simplicio's mouth, and that that was the real cause of the trouble into which Galileo fell. The last great book that I can recall in dialogue form is Boyle's *Sceptical Chymist*.

Books that have made scientific history will readily occur to everyone. As examples we may take Linnaeus's *Genera Plantarum*, Lagrange's *Mécanique Analytique*, Lavoisier's *Traité Élémentaire de Chimie*, Carnot's *Puissance Motrice du Feu*, Liebig's *Organische Chemie*, and in more modern times Clerk Maxwell's *Electricity and Magnetism*, J. J. Thomson's *Conduction of Electricity through Gases*, Rutherford's *Radioactivity*, Sherrington's *Integrative Action of the Nervous System*, and Rayleigh's *Sound*. You will have to consider, I am afraid, the book and no doubt you will be urged from some quarters that all books ought to be the same size. It would probably save wood in bookshelves, or steel, and is, I suppose, by no means a matter to be hastily dismissed by questing minds. Some forward-thinking spirits may further suggest that they should all be printed by the same one publishing house, which by due coordination, a word to which Professor Rocard has recently devoted a learned treatise, would—should, rather—lead to great economies, as production would then be properly planned.

There are certain questions of the modern production of scientific books which are raised by the study of the old books of science. The beauty of the old production we can scarcely hope to imitate nowadays: craftsmen like those who, cutting the woodblocks for geometrical diagrams, found the work dull and so filled in the blank spaces with elegant little designs, producing the so-called floriated diagrams, no longer exist. Our materials will not allow us to use the device adopted in Newton's *Opticks* and many books of Newton's time\* to make diagrams visible to the reader without turning back while he reads the several pages that contain references to them, namely, to print the diagrams on a folding plate with a blank space, the size of a page, where it is attached to the book, so that when the plate is extended and the book closed the whole engraved part of the sheet is still visible. For one thing, our paper would not stand the repeated folding and unfolding. Descartes solved the same problem of enabling the reader to have always before him a diagram to which reference is made on several pages, by reprinting it on every relevant page—thus in his *Principia* one particular diagram is printed eleven times. We have not the paper for this extravagance.

The old books raise other questions of illustration. They used, in the best period, the woodcut and the copperplate: illustrations such as those to Senguerd's

\* The earliest example that occurs to me is Lana's *Prodromo*, of 1670, but quite likely this is not the first.

*Philosophia Naturalis* are little works of art, worthy to be compared with Della Bella's fine frontispiece to Galileo's *Dialogo*. Many aspects of the graver's art appear: thus the illustrations to Thomas Wright's *New Hypothesis of the Universe* (1750) are in mezzotint, which beautifully renders the soft blackness of the night against which the stars shine. To-day we have neither time, money, paper nor craftsmen for illustration on the lines shown in early books, but we have something which our ancestors lacked, photography and the various photographic processes of reproduction. Photography, it must be remembered, is a convention just as much as line drawing: what is produced is a version of the object or phenomenon

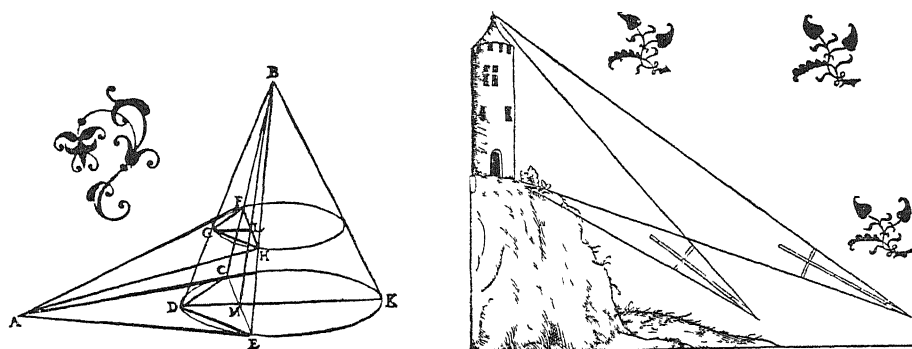


FIGURE 2. Floriated diagrams.

photographed, and in no sense a true, in the sense of a unique, representation. To realize this, one has only to think of the effect of the direction of illumination on what is seen in a microphotograph of a metal surface, say, or of the differences that can be produced by colour filters and different types of photographic plate in rendering any subject. The use of the photograph has, of course, transformed observational astronomy and bacteriology, and no book on these subjects can be without photographic illustrations. It would be out of place to attempt to trace the manifold uses of the photographic illustration, but it may be permissible to point out that there are many purposes for which a good drawing is preferable. Photographs of instruments and complicated installations of apparatus are seldom successful: it is nearly always inessentials that are prominent in them and awkward shadows have a way of hiding what is wanted. I venture to give here a reproduction of a good photograph of an experimental set-up (figure 4, plate 12): essential features, as, for instance, the form of the arm just above the vessel at the bottom of the picture, hardly tell at all. This should be contrasted with any good line illustration of apparatus, either in old or newer books.\* Perhaps I may quote here what Fuchs says in 1542 in the preface to his *De historia stirpium*: 'Furthermore we have purposely and deliberately avoided the obliteration of the natural form of the plants by shadows, and other less necessary things, by which the delineators

\* In my lecture I took as an example of the confused masses frequently shown in photographs of apparatus a picture from a published book; this was much worse than the one here reproduced, which is from a photograph taken in my own laboratory. I can, however, hardly ask the publisher to allow me to reproduce it as a warning.

sometimes try to win artistic glory: and we have not allowed the craftsmen so to indulge their whims as to cause the drawing not to correspond accurately to the truth.' For the representation of plants in their surroundings, ecology, photography is often excellent: for the representation of structural botany the illustrations of a hundred years or more ago have never been equalled. Photographic reproductions need special, so-called art, paper, which is unsuitable for ordinary book pages—it is too heavy and does not make a good page. Incidentally, the weight of books is a matter worthy of some discussion—a very distinguished man of science has protested to me about the great weight of some single volumes printed on art

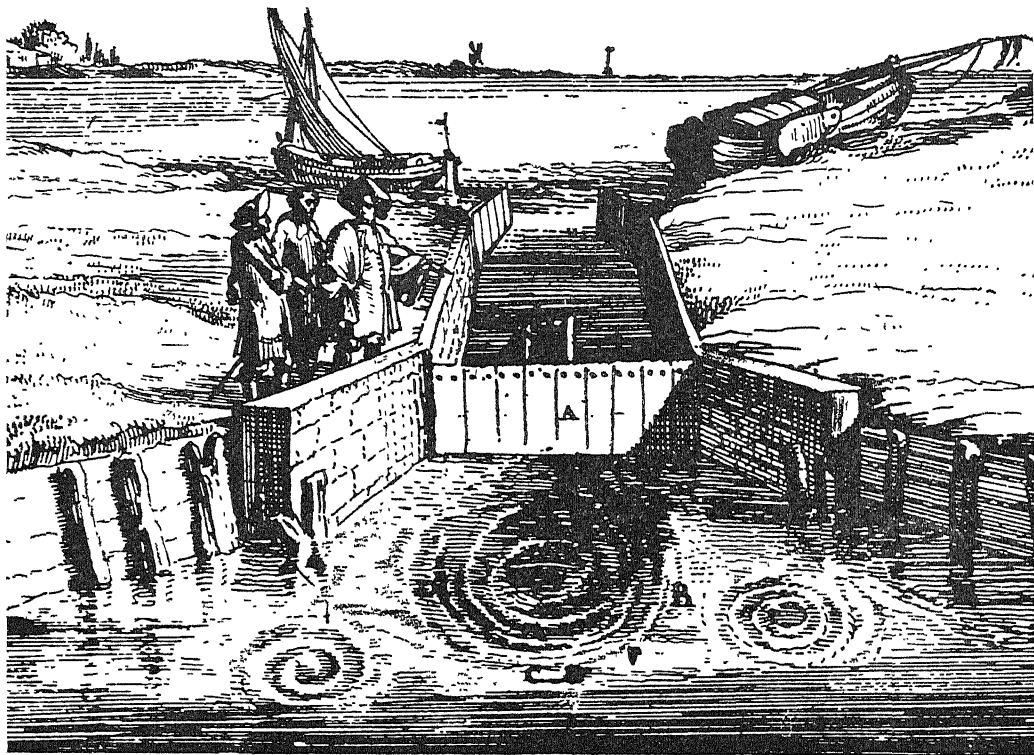


FIGURE 3. An illustration, by Schoonebeck, from Senguerd's *Philosophia Naturalis*, 1681.

paper and quoted a recent American publication which weighs about  $4\frac{1}{2}$  lb. Certain German books in which sections of art paper are introduced among normal paper and printed as ordinary pages have an unpleasant appearance. The whole question of the illustration of scientific books and periodicals is worthy of consideration, and clearly cannot be without influence on format and make-up.

If the old books and journals can offer us an example in many matters of material production, which we may be kept from following by material dearth, they are no less a pattern in certain matters of literary presentation. I would not suggest, of course, that the form of Newton's *Principia* is one that we should imitate to-day, although Thomas Young set out his Bakerian Lecture, in which he developed the wave theory of light, in *Propositions* and *Corollaries*, somewhat in the Newtonian method. It would be vain to wish to hear again the sonorous swing of Georgian

prose in science, although rebukes such as 'We can only regret that a person so void of a sense of physical elegance should have an opportunity of obtruding opinions like these on the public' or 'The reviewer has afforded me, in the next observation, an opportunity for a triumph as gratifying as any triumph can be where the enemy is so contemptible' would add to the interest of scientific controversy. I could, however, wish that the clarity and elegance with which so many of the older writers set out their thoughts and experiments would find more imitators to-day. The great papers of J. J. Thomson and of Rutherford have a straightforward lucidity of phrase. When, as a young man, I had to learn something of the calculus of variations, someone suggested that I should read Lagrange's original memoir, and I still remember the pleasure that his exposition gave me. Thomas Young, whom I have just quoted, sets a good example, but he was a great classical scholar, bred in the traditions of literary style. Faraday, however, was practically untaught—'My education was of the most ordinary description, consisting of little more than the rudiments of reading, writing and arithmetic at a common day-school'—and yet his writing of matters sufficiently abstruse shows a beautiful clarity and simplicity. Consider the following passage from his paper in the *Philosophical Magazine* entitled 'A Speculation touching Electric Conduction and the Nature of Matter':

But it is always safe and philosophic to distinguish, as much as is in our power, fact from theory; the experience of past ages is sufficient to show us the wisdom of such a course; and considering the constant tendency of the mind to rest on an assumption, and, when it answers every present purpose, to forget that it *is* an assumption, we ought to remember that it, in such cases, becomes a prejudice, and inevitably interferes, more or less, with a clear-sighted judgment. I cannot doubt but that he who, as a wise philosopher, has most power of penetrating the secrets of nature and guessing by hypothesis at her mode of working, will also be most careful, for his own safe progress and that of others, to distinguish that knowledge which consists of assumption, by which I mean theory and hypothesis, from that which is the knowledge of facts and laws; never raising the former to the dignity or authority of the latter, nor confusing the latter more than is inevitable with the former.

Light and electricity are the two great searching investigators of the molecular structure of bodies...

This deals with a matter which nowadays is often smothered in a complexity of long words and cumbrous and ambiguous phrases, yet here the sense is clearly and unmistakably expressed, whether some of our moderns would agree with it or not.

I cannot help feeling that much of the obscurity that hangs about many papers to-day is unnecessary, and I am in good company in objecting to it, for Lord Rayleigh records of J. J. Thomson 'Like Lord Kelvin he was in general impatient of obscurity'. I am not thinking of our Newtons: they must go their own way, although we may note that William Molyneux writing to Hans Sloane in 1697 said: 'I hear Mr. Newton's *Phil. Nat. Prin. Math.* is out of press, and that he designs a 2nd Edition. Pray advise him to make it a little more plain to Readers not so well versed in Abstruse Mathematicks, a few Marginal Notes and references and Quotations will doe the business.' The gentlemen whom I find tiresome are those who write matter that is unintelligible not on account of its difficulty but on account of the confusion of thought, the lack of sequence and the tangled, sometimes ungrammatical jargon in which it is expressed. Again, there are certain writers of

papers on mathematical physics whose productions are difficult to appreciate because the assumptions which have been made are never clearly expressed, but introduced casually by the application of formulae designed for other circumstances. Others have an annoying habit of complicating matters by introducing a spurious and superficial generality which is thrown away as soon as the real problem which is under treatment is approached. It would save the reader much trouble if every writer of a scientific paper would first state clearly what he has done and why it is of interest: if a mathematician would say exactly what he has assumed, what method he has applied and what results obtained. I have sometimes wondered if certain important mathematical papers dealing with physical problems would not have a much wider circle of readers—and of readers who would profit—if much of the work were relegated to a kind of mathematical appendix, the writer saying that from one stage he has passed to the next by the process described in this appendix. I realize that I am laying myself open to envenomed attack, but the attackers may even learn something in sitting down to explain just how absurd my suggestion is.

Many who realize how much some of the papers written to-day, especially as first presented, are wanting in clarity and conciseness, to say nothing of elegance, have suggested that our young research workers should be made to take courses in English in the English departments of our universities. I scarcely think that this is the way to go about it. Some of the worst English that I have read has been written by D.Litt.s, and I used to cut out the writings of a certain headmaster that occasionally appeared in the newspapers to keep them as examples of turgid language. Rather read to the young man an account of a piece of work as written by a master and then let him write his own account and compare it subsequently with the original. Personally I feel that a wrong ideal is often the cause of much of the trouble. The young man wants a long paper suspecting, in many cases rightly, that when it comes to applying for a higher post his achievement will be estimated by the number of pages that he has produced. He wants one that looks as formidable as possible judging, in many cases rightly, that the senior who looks at it will think more highly of him if it is full of forbidding phrases and long words, especially if they are new words, such as, let us say, diclastically disintegrated to mean 'cut into two bits'. If it were conveyed to him that simple expression is in itself desirable, that every new word needs careful justification, that scientific jargon is not in itself scientific advance there would, I think, be an improvement. Personally, when a young man brings me a mass of verbiage I ask him, gently, what exactly he means, and when, as usually happens, after much questioning he has reached a perfectly simple form of words I say 'Then why not say so?' I think that it is as simple as that. But, to use the language of to-day, I am sticking out my neck. Perhaps I may save myself some blows in this vulnerable region if I insist that I realize that everybody cannot write great and memorable prose like that of Charles Sherrington—but then he is a fine poet and for fine poets words have a ring, a friendly aptness like that which his tools have for the master craftsman, which they do not have for the actuary. All that I ask is a freedom from unnecessary clumsiness and obscurity. I do not ask that scientific papers shall be pleasant to read, only that they shall not be repugnant.

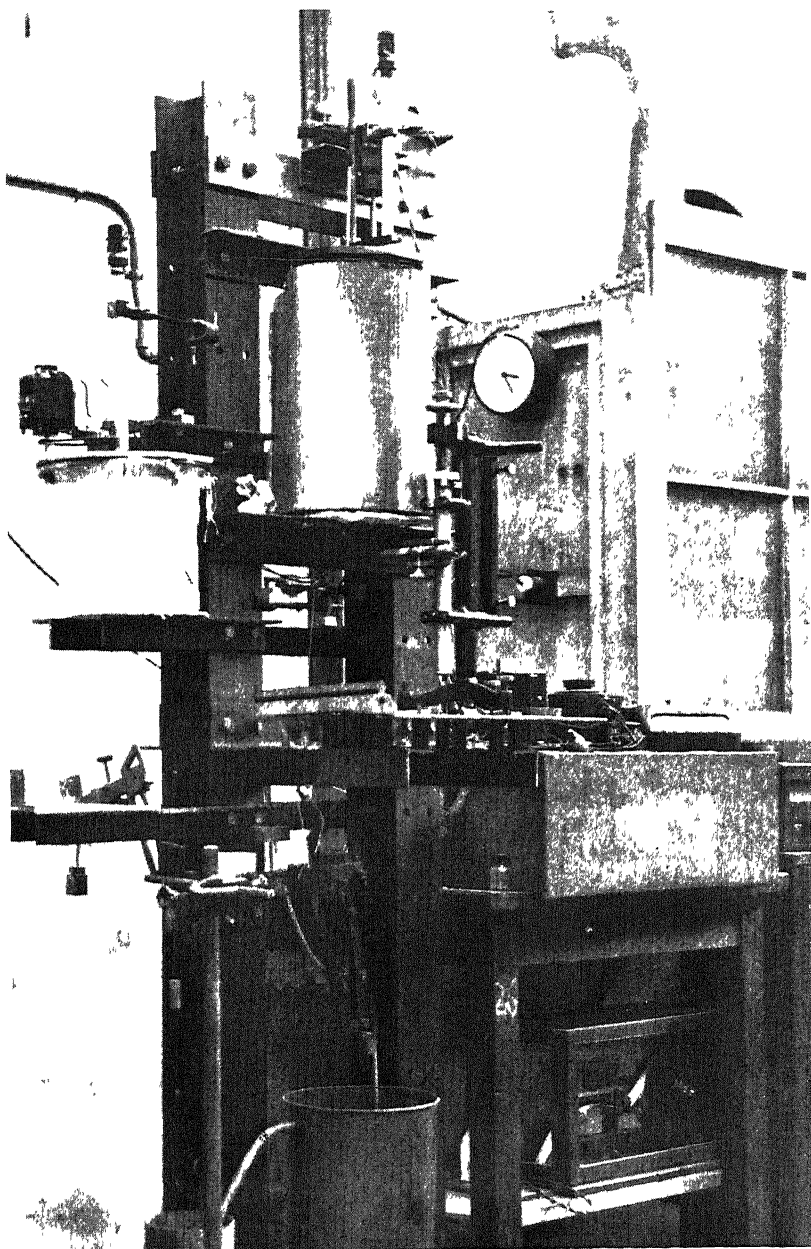


FIGURE 4 A photograph of a simple assembly of apparatus





There is to-day a growing specialization in science which is inevitable. We no longer have men of learning writing for other men of learning, or men of science writing for other men of science, or disciples of exact science writing for other disciples of exact science, or physicists writing for other physicists, or students of optics writing for other students of optics, or spectroscopists writing for other spectroscopists, but rather infra-red spectroscopists writing for other infra-red spectroscopists—and very soon they will have a journal of their own. You, gentlemen, are dealing with the very complex problems that such a situation creates, and I am confident that great simplification and great economies will result from your labours, and that the toil of the specialists in the smaller and smaller regions of greater and greater complexity will be lightened. But the great advances will still be made by solitary spirits who have greatly pondered on more general problems, who have, like Newton, kept the subject constantly before them until the first dawns open little by little into the full light. Let us take care that we do not organize things to such a point that no leisure exists, since, for the pioneers, leisure is even more needed than journals, reference books and resolutions.

*For certain periodicals which are in an unfamiliar language, e.g. Swedish, I have given the English equivalent of the title. Readers who want the actual title can find it in the list of periodicals given at the end of any edition of the Landolt-Bornstein tables.*

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## The work of the National Institute for Medical Research

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*Lecture delivered 10 March 1949—Received 21 March 1949.)*

[Plates 13 and 14]

In the National Insurance Act of 1911 there was contained a provision which has proved to be of great importance for scientific work in this country. This provision laid down that the sum of one penny per insured person should be provided from public funds for the purposes of research. The total income resulting amounted to £55,000 in the first year of the operation of the Act, and it was for the administration of this sum and for the decision as to the purposes to which it should be put that the Medical Research Committee was appointed in 1913.

The first report of the Committee, which appeared late in 1915 over the signature of the first secretary, Walter Fletcher, and was submitted to the Chairman of the National Health Insurance Joint Committee, contained a declaration of policy which is of such fundamental importance to the conduct of medical research that it

seems to me to be worth quoting in full at this time. In defining their objects the Committee made the following statement:

'The object of the research is the extension of medical knowledge with the view of increasing our powers of preserving health and preventing or combating disease. But otherwise than that this is to be the guiding aim, the actual field of research is not limited and is to be wide enough to include, so far as may from time to time be found desirable, all researches bearing on health or disease, whether or not such researches have any direct or immediate bearing on any particular disease or class of diseases, provided that they are judged to be useful in promoting the attainment of the above object.'

I shall have occasion later to refer to researches which have been carried out at the National Institute for Medical Research, and which, although they have proved in the outcome to be of importance to medicine, were in their inception purely scientific inquiries. It is clear that only within the framework of the almost academic freedom claimed in the statement of policy which I have read could such researches have been undertaken in an Institute for Medical Research.

Having laid down their general policy in these terms the Medical Research Committee proceeded to consider the steps to be taken for its implementation; the methods decided on were four in number, namely, (1) the employment of full-time investigators in centralized laboratories, (2) the appointment of full- and part-time investigators in hospitals and elsewhere for special researches, (3) the provision of assistance grants to workers in academic institutions engaged in independent researches in the general field of interest, and (4) the maintenance of a statistical department. The individual importance attached to the last of these items must be ascribed to the obvious bearing of statistical work on the problems raised by national insurance; actually this item was merged with the first, which itself constituted a decision to establish a research institute.

Very soon after the decision had been taken to set up a central research institute, which, incidentally, was to be paid for out of the first year's income of the Medical Research Committee, negotiations were started for the purchase of the Mount Vernon Hospital, Hampstead, with its freehold site and grounds, at the price of £35,000, it being considered, in the words of the report, that these premises offered 'exceptional advantages in amenity and convenience' besides being readily convertible into research laboratories; in addition, it was at this stage intended that part of the building should be used as a research hospital of fifteen to twenty beds.

Whilst these negotiations were in progress a proposal was made to the Medical Research Committee by the Governing Body of the Lister Institute, that the latter should, subject to the agreement of the members and to satisfactory provision for the existing staff, be handed to the nation for use as the central research institute of the Committee. The importance which was attached by the Medical Research Committee to the research hospital part of their project is shown by the fact that the first check in the negotiations with the Governing Body of the Lister Institute arose because the latter possessed no hospital; this difficulty was overcome by the generous offer of Lord Iveagh to build a fifty-bed hospital on a site adjoining the Lister Institute. Moreover, the Medical Research Committee were prepared to take

into their employment the whole of the existing staff of the Lister Institute. However, when the proposals were finally submitted to the members of the Lister Institute for approval they were rejected and the scheme came to nothing.

So long as the result of the negotiations between the Medical Research Committee and the Governing Body of the Lister Institute remained in doubt it was naturally impossible to proceed with the work of converting the buildings at Hampstead, the result of this delay was that the outbreak of war in August 1914 found these buildings untouched, and in October of that year it was decided to hand them over to the War Office for use as a military hospital. Thus the control of the Hampstead premises passed out of the hands of the Committee for five years, although much work was done on these premises during that period in which the Medical Research Committee had a direct interest and in which members of their staff took a leading part. When the Mount Vernon Hospital was handed to the War Office it was selected for the study of cardiac disorders—the so-called ‘soldier’s heart’—and the direction of this work was undertaken by Thomas Lewis, who was seconded to Hampstead from University College Hospital, where he was already working as a full-time member of the Committee’s scientific staff. The work on cardiac disorders was continued at Hampstead until 1917 when it was transferred to Colchester, and the Mount Vernon Hospital then became a Central Hospital for Flying Officers; a considerable amount of work was done on the special physiological and medical problems raised by the stresses of flying, and in this again the Medical Research Committee took their part.

In preparation for the staffing of the central institute the Medical Research Committee had already, during 1914, made certain key appointments for the three main divisions of the work which were at that time envisaged. These appointments were those of Sir Almroth Wright and S. R. Douglas in bacteriology, of H. H. Dale, G. Barger and A. J. Ewins in pharmacology and biochemistry, and of Leonard Hill, Benjamin Moore and Martin Flack in applied physiology; the department of statistics, which was also to be housed in the central institute, was put in the charge of John Brownlee. Since the Hampstead building was not available these workers had to be accommodated elsewhere, and during the war the bacteriologists were based on St Mary’s Hospital, although much of their time was spent in France, the pharmacologists and biochemists were at the Lister Institute, and the applied physiologists at the London Hospital; the department of statistics occupied a house in Guilford Street, Russell Square.

The work of the scientific staff of the potential institute during the years 1914–19 was inevitably almost entirely concerned with problems raised by the war; this work I can only mention in passing. Sir Almroth Wright and S. R. Douglas, later joined by L. Colebrook and Alexander Fleming, were engaged on a continuous study of wound infections and dysentery. The pharmacologists and biochemists led by H. H. Dale worked on shock, on trinitrotoluene poisoning and, in conjunction with Clifford Dobell, on amoebiasis; Dale, in particular, also gave much of his time to the control of drugs, especially the arsenical chemotherapeutic agents, and this work was of great importance in relation to later developments of biological standardization to which I shall have to refer. The applied physiologists were occupied with

studies of environmental hygiene, and later in the war took part in the research on aviation problems in which Martin Flack was closely concerned.

Possession of the Hampstead building was regained by the Medical Research Committee in June 1919, and work was begun on its conversion into laboratories in the anticipation of occupation by November of that year. Actually, although it was possible to instal the statistics department at an early stage, occupation by the laboratory workers was delayed until April 1920. Even then the equipment was incomplete, and it is recorded that only 'minimum decorative completion' was attempted. During the process of conversion of the hospital into laboratories the nurses' home adjoining the main building was acquired by the Committee and let to the head of the department of biochemistry and pharmacology, H. H. Dale.

In 1920 the Medical Research Committee was reconstituted under grant of a Royal Charter as the Medical Research Council, a body responsible to the Committee of the Privy Council for Medical Research, of which the Lord President of the Council is Chairman. In the Report of the Council for that year the establishment at Hampstead appears for the first time under its present name of the National Institute for Medical Research; it is noteworthy that by this time the plan for the incorporation of a research hospital within the Institute on which so much stress had previously been laid had completely disappeared, although no reference to its formal abandonment is to be found in the Reports of the Council. Reference is made in 1920 to the Institute in the following terms:

'The research work which has been in progress there in all the departments during the past six months has already proved, as the Council think, that the building, though constructed for hospital purposes, is admirably suited for the purposes now in view. This working experience has fully justified the original choice of this building made by the Medical Research Committee in 1914. With very little structural alteration the rooms have provided convenient and effective laboratories. The workers enjoy almost complete immunity from fog during the winter months, while the building and grounds, though within two minutes walk of the Hampstead Tube Station, are free from the noise of traffic and from electric or other disturbances outside.'

Although my present colleagues who have had to work under conditions of some overcrowding and discomfort during the last few years in the Institute may regard this as a somewhat rosy picture, there is no doubt that in fact the Hampstead building did serve its general purpose admirably for a long time and that the advantages of its situation were not overrated. Nevertheless, unforeseen developments of work with special requirements soon made further provision necessary, and in 1921 the Council purchased a site at Mill Hill where farm laboratories were erected for investigations of viruses which could not be accommodated at Hampstead. Moreover, the value of the Hampstead building itself was greatly increased by the addition in 1928 of an animal house, the cost of which was provided from funds bequeathed to the Council by the late Lord Justice Ronan.

The additional accommodation made available by the construction of the Ronan Building, and by the removal of the Department of Statistics to the London School of Hygiene which occurred in 1927, made the total laboratory facilities at Hampstead

adequate until the decision of the Government in 1936 to allocate an additional sum of £30,000 a year to the Council for development of research in chemotherapy raised a new demand for expansion. Since it appeared that such expansion could not be satisfactorily carried out on the Hampstead site the Council made the important decision to erect a new and larger building on their property at Mill Hill to the acquisition of which reference has already been made. This new building was to be designed to house all the existing activities at Hampstead and to provide for the necessary expansion of work in chemotherapy and in other fields of growing importance.

Work on the new building was begun in 1937, and by the summer of 1940 the main structure was complete. At this time, owing to the situation created by the war, the work had to be interrupted and the building was lent by the Council to the Admiralty, by whom it was used as a training establishment for the W.R.N.S. until April 1945. Since then, with many delays, the work of completion of the building for the purposes for which it was originally designed has proceeded, and is now nearly finished, so that from a physical point of view the Institute at present stands on the threshold of the greatest development in its history.

The organization of the Institute in its early days was somewhat peculiar. We have seen that in the appointment of scientific staff the Council had selected leaders in three main branches of laboratory work, in addition to statistics, which stands outside the present discussion. These senior members of the staff each had a department, but there was no Director. Each of the heads of departments, and, indeed, all other members of the scientific staff, were regarded as being directly responsible to the Council through the Secretary.

As might be expected, such an arrangement ultimately proved inconvenient in practice, and in 1928 it was revised by the appointment of H. H. Dale as the Director of the whole Institute. Nevertheless, the idea which lay at the back of the initial organization was an important one; this was that rigid division of the Institute into departments was at all costs to be avoided. The importance of maintaining free intercourse between workers in the different laboratories at the Institute was repeatedly emphasized in the earlier Reports of the Council. It has remained a guiding principle in the administration of the Institute to the present day, and I sincerely hope that it will continue to do so in the future.

Turning now to the actual scientific work of the Institute we have first to notice that the staff which finally assembled at Hampstead in 1920 was different in several important respects from that which had originally been appointed. At the end of the 1914-18 war Sir Almroth Wright and Alexander Fleming returned to St Mary's Hospital, and George Barger was appointed to the Chair of Medical Chemistry at Edinburgh, whilst A. J. Ewins had already taken up industrial research work. When the Institute opened therefore the three laboratory departments were headed by S. R. Douglas, H. H. Dale and Leonard Hill respectively; important accessions to the staff at this time or within the next year or two were W. E. Gye, Clifford Dobell and P. P. Laidlaw in pathology, H. W. Dudley and Harold King in biochemistry and pharmacology, and Percival Hartley, who later took charge of the work on biological standards.

For the first year or two at Hampstead senior members of the staff were chiefly occupied in winding up their researches of the war years, but soon several main lines of new work began to emerge, and it is on the discussion of some of these main lines that I must base my account of the scientific work of the Institute. One of the earliest researches to be begun was that on virus diseases; since this has remained a major theme at the Institute until the present time, and since the development of the work has some points of general interest I propose first to give this some attention.

The importance of virus diseases in human medicine coupled with the ignorance prevailing at that time concerning the viruses themselves were the reasons for the decision to begin systematic investigations of the general problem. The first attempts were directed towards the cultivation of viruses *in vitro*; these having failed, attention was turned to a naturally occurring animal virus disease, namely, dog distemper, which promised to afford a direct experimental approach. With the support of funds raised by the *Field* newspaper, a study of dog distemper was therefore undertaken, the leader of the investigation being the late P. P. Laidlaw with collaboration on the veterinary side from G. W. Dunkin who was appointed to the staff for the purposes of this work. Early on in the study a discovery which later proved to be of major importance was made in the scientific proof that the ferret was susceptible to distemper, a fact which had long been a matter of popular belief. This meant that a convenient experimental animal was available in which the disease could be produced at will, and the conditions were thus provided for a close study of the biological behaviour of the infective virus. In the course of a few years' work sufficient knowledge was accumulated to make possible the production of an effective vaccine against distemper, with the aid of which, as is now well known, the disease in dogs can be prevented.

The problem of dog distemper having been solved, some time was spent in the study of various other viruses which could be attacked experimentally, including the bacteriophages which were then suspected and are now generally recognized to be viruses parasitizing bacteria. Then in 1933 came the outstanding discovery by Laidlaw, Andrewes and Wilson Smith of the influenza virus. This discovery was made by a return to the technique of the dog distemper work, the ferret again proving to be susceptible to the disease and providing the means for experimental study of the virus. Since that time of course various other techniques for the experimental investigation of viruses have been developed, most notably the method of cultivation in developing eggs first discovered by Goodpasture in the United States and later improved by F. M. Burnet working in the Institute, and there is no doubt that these techniques have greatly extended the possible range of investigations. Nevertheless, it can reasonably be claimed that the work on dog distemper followed by that on influenza really opened up the whole field of precise study of virus diseases in animals and man.

One piece of work which at first formed part of the general virus investigation and which deserves special mention is that of W. E. Gye on the virus factor involved in the production of transmissible tumours. Although all the perplexities of the problem have not been resolved, this work, since greatly extended by Gye himself

under other auspices and by many workers elsewhere, together with the independent contribution made to it by C. H. Andrewes, has undoubtedly had a profound influence on cancer research, and it is a matter of pride that it was initiated at the Institute.

The development of knowledge of the biological and epidemiological properties of viruses which resulted from the investigations which I have mentioned naturally led to the desire for more information about their physical properties. Of these the one which clearly first required attention was the actual size of the virus particles, and this problem was attacked in two ways, namely, by the development of quantitative methods of ultrafiltration and by the refinement of optical technique. The work on ultrafiltration resolved itself into a study of the mode of preparation and of the behaviour of standardized collodion membranes having average pore sizes within closely defined limits; by the use of such membranes which were developed by W. J. Elford in the course of a number of years of work and which are now in common use, it was possible to form an estimate of the size of the particles of a virus by ascertaining the minimum average pore size of a membrane which would allow the virus to pass. In this way the sizes of the particles of a number of viruses were assessed, and the values found have in many cases been confirmed by the use of other physical methods since developed.

The particles of most viruses are of course so small that they cannot be observed microscopically with the ordinary microscope; in order to obtain the degree of resolution required it is necessary to use light of wave-lengths shorter than those of the visible part of the spectrum. The use of ultra-violet light offered obvious possibilities in the direction required, and much effort was expended by J. E. Barnard at the Institute in the development of a satisfactory system of ultra-violet microscopy. The effort put into this work was richly rewarded, not only in the results obtained in the observation of viruses for which it was originally carried out, but in the foundation which it laid for the modern cytological work which, in many laboratories throughout the world, is doing so much to unravel the chemical nature and the biochemical behaviour of the cell nucleus.

The advent in recent years of the electron microscope, with a power of resolution of a different order from that of any optical instrument, has provided another powerful tool for the study of minute objects. Full use has been and is now being made at the Institute of the electron microscopical technique for the examination of viruses. With the aid of new methods of isolation and purification of viruses, and improvements in the method of preparation of specimens, the use of the electron microscope seems indeed to be placing the study of the morphology of viruses upon a new plane.

Whilst great advances have been made in many laboratories in the acquisition of general knowledge about the behaviour and properties of viruses since the beginning of the work at the Institute, it cannot be claimed that progress with the practical treatment of the diseases which they cause has been equally rapid. Indeed, a new advance in this part of the field is the outstanding need of the present. Recent partial successes in the United States, for instance, the discovery of antibiotics such as chloromycetin which cures certain rickettsial diseases and aureomycin

which may be active against some true virus infections as well, seem to promise that the advance will come from chemotherapy, and this aspect of the problem is one which is naturally attracting a good deal of attention at the Institute as elsewhere.

Apart from the direct scientific and medical significance of the work on viruses about which I have been speaking, the progress of development of the work and the way in which the problem as a whole has been tackled, have, I think, some points of general interest to which I would like to refer. When the investigation was started it was naturally regarded as essentially a medical problem, and the work was therefore initiated by men whose experience lay in the fields of bacteriology and human pathology. As soon as it appeared that the direct attack was unpromising the direction of effort was diverted to an animal disease, the necessary expert help being obtained by the inclusion of a veterinary surgeon in the group responsible for the research. Again, as the general programme progressed, further additions were made for extensions of the work on the physical and optical sides. In each case the new men who joined the group did so as equal partners, and with freedom simultaneously to develop their own ideas and lines of investigation, which they did with good effect.

There is nowadays a good deal of discussion as to the part which can usefully be played by teams in research. It seems to me that the history of the virus work at the Institute which I have just described forms a good illustration of effective team work in medical research, although at no time has there been any pretence at the formal constitution of a team. It is perfectly clear that the field of scientific effort covered by the research as a whole has been so wide that no one man unaided could have made much progress. On the other hand, at the outset of the investigation it was quite impossible to predict the devious paths which would have to be followed and the different branches of science into which excursions would have to be made. In these circumstances the course which was actually followed seems to have been the best, the essential feature being readiness at all times to extend the investigation in any desired direction by the addition of the help of an appropriate worker. In this way there is in fact ultimately formed a team of investigators whose efforts are all generally directed to the same end; but the team is never rigidly defined; it grows only in response to the demands of the work, and the individual members retain a considerable degree of scientific independence and scope for initiative. I am myself convinced that, for the organization of work on problems requiring co-operative effort for their solution—and this includes many problems of medical research as it has developed to-day—these are the correct principles.

This, however, is a digression, and I must return to the main theme of the scientific work of the Institute. The next line of investigation to which I wish to refer, particularly because it has been mainly associated with the name of the first Director, to whom it eventually brought the honour of the Nobel Prize for Medicine, is one which in its various ramifications has occupied the attention of a large proportion of the scientific staff of the Institute for many years. In general, it may be defined as the study of the chemical control of bodily functions, and, in fact, it



has been mostly concerned with the biochemical and physiological investigation of two compounds, histamine and acetylcholine.

The first of these had long been known as a substance with intense physiological activity, which occurred in nature as a constituent of ergot and as a metabolic product of the action of certain bacteria on the commonly occurring amino-acid histidine. Several of the effects produced by the administration of histamine were closely similar to those observed in anaphylactic shock, and largely as the result of work by Dale it had come to be assumed that the phenomena of anaphylactic shock and of allergy in general were actually due, at least in part, to the liberation of histamine. It also appeared, both from observations by the late Sir Thomas Lewis on the nature of the response of the skin to injury, and from further physiological studies of histamine carried out in the Institute, that liberation of this compound, with its known power of dilating capillaries and causing changes in their permeability, might be responsible for many of the local reactions of tissue to trauma.

These suppositions naturally implied the existence in normal tissues of a source of histamine, a substance which had so far only been found in the mammalian organism in a situation in which it was presumably a product of bacterial action. In parallel with the physiological studies therefore a systematic search for histamine in various normal animal tissues was begun by H. W. Dudley in the Institute, and led to the discovery that histamine in a bound form was in fact distributed quite widely in animal tissues, in some of which, particularly lung, it occurred in surprisingly large amounts. Thus the objective evidence was supplied which was necessary to establish the supposed role of histamine in producing the phenomena of anaphylactic shock, allergy, and local tissue reaction to injury.

In the course of the biochemical work on histamine a discovery was made which turned out to be of great physiological importance; this was the observation that extracts of normal spleen contained small amounts of the highly active substance acetylcholine. This compound was hitherto known in nature only as a constituent of ergot. Sufficient information had, however, already been acquired about its physiological actions to suggest that either acetylcholine itself or a substance closely resembling it might be concerned in the production of the effects of stimulation of the parasympathetic nerves. The demonstration of the actual occurrence of acetylcholine as a normal constituent of the body naturally gave a great stimulus to this idea which was actively developed by Dale with a succession of colleagues including J. H. Burn, J. H. Gaddum, W. Feldberg, G. L. Brown and F. C. MacIntosh, in work extending over many years at the Institute and by workers elsewhere. The details of the investigations are not for discussion here; the great generalization to which the work led may, however, be recalled: this is the conclusion that acetylcholine is the substance responsible for the transmission of nervous stimuli from the peripheral ends of parasympathetic nerves to plain muscle, through the ganglionic synapses of the sympathetic nervous system, and at the nerve endings of motor fibres to voluntary muscle; that is to say, it is the chemical transmitter for the effects conveyed by the whole of the efferent fibres of the peripheral nervous system except the postganglionic fibres of the sympathetic system for which a

similar part is played by adrenaline. Not only did this generalization form a new chapter in neurophysiology, but the implications of the work as a whole for neurological medicine have been highly significant.

Turning to another series of physiological and biochemical researches, the subject of carbohydrate metabolism in its various aspects is one which has attracted a good deal of attention at the Institute. The interest aroused by the first announcement in 1922 of the discovery of insulin at Toronto caused the Council to send H. H. Dale and H. W. Dudley to Canada in order to report on steps which should be taken to ensure the rapid and proper development of the discovery so far as this country was concerned. The immediately important result of this mission was the acceptance by the Council of the British rights in the patents covering insulin manufacture, and the employment of the control given by these rights to establish British production of insulin on a scientifically sound basis.

A contribution to the method of purifying insulin was made at the Institute which was of some considerable use in the early stages of its manufacture in this country, and the methods for its biological assay were studied in detail in connexion with the control of the quality of the product sold to the public. Apart from this, however, the availability of insulin stimulated interest in the details of the hormonal control of intermediate carbohydrate metabolism. In the course of some years experiments were carried out which revealed several of the underlying facts determining the utilization of carbohydrate in the animal body. A later development arose from the study of the role of hormones produced by the anterior pituitary gland in the control of carbohydrate metabolism, and this eventually led to what is probably the most important discovery in this particular field of work to be made in the Institute, namely, the observation by F. G. Young that it was possible by purely hormonal means to produce experimentally in dogs and cats a permanent condition of diabetes indistinguishable from the natural disease as it occurs in man.

Before leaving the subject of biochemistry there is one other investigation which I should like to mention, since the results of the work will always be associated with the name of the Institute; this is the research work which led to the identification of one form of vitamin D—that which is now known as vitamin D<sub>2</sub>. The whole study was again pre-eminently an example of co-operative research; it started with an inquiry into the nature of the process by which vitamin D activity was conferred on sterols by irradiation with ultra-violet light; this inquiry revealed the identity of the provitamin with ergosterol and opened the way for experiments on the direct irradiation of this compound which ultimately led to the isolation of pure vitamin D<sub>2</sub>, or, as it was called, calciferol. This work is a further example of a research carried out by a team which grew during its progress; having been initiated by O. Rosenheim and T. Webster it was continued with collaboration, among others, from R. B. Bourdillon on the physical side, and in its organic chemical and biochemical aspects from R. K. Callow and Miss H. M. Bruce.

Apart from this detailed study of one vitamin, and a contribution on the nutritional significance of phytic acid in cereals, nutritional research has not been undertaken to any considerable extent within the Institute itself; on the other hand, in

the associated Nutrition Laboratory a continuous programme of such work has been maintained, amongst the results of which we may recall the investigation of abnormalities of bone growth associated with vitamin A deficiency which formed the subject of Sir Edward Mellanby's Croonian lecture to this Society in 1943, and the recently much discussed discovery of the toxic effects of 'agenized' flour.

The Medical Research Council have always shown themselves to be fully conscious of the contribution which chemistry can make to medical research. We have already seen the part which chemists and biochemists have played as collaborators in several of the major themes of research which I have mentioned. They have, however, also made their own independent contributions, and particularly in the development of chemistry in relation to pharmacology and chemotherapy. We may recall, for instance, two advances which have resulted from a large amount of work on alkaloids. The first of these arose out of clinical observations by Chassar Moir at University College Hospital; it led to the isolation by H. W. Dudley from ergot extract, at a time when it was thought that everything was known about the alkaloidal content of this material, of a new alkaloid which actually turned out to be the really important constituent of ergot from the point of view of its practical use in medicine. The second is the work of Harold King on curare alkaloids which has led to the isolation and chemical identification of the pure alkaloid D-tubocurarine which is individually responsible for the typical physiological action long associated with curare. This work also has had its therapeutic application in the extensive use which has been and is still being made of D-tubocurarine as an adjuvant in anaesthesia to facilitate the task of the surgeon by providing more complete muscular relaxation. As an extension of the investigation of curare an interesting advance has been made quite recently which again may have practical application in medicine; this consists in the developments by chemists and pharmacologists working in collaboration, of a series of simple bisquaternary salts, one of which promises to be therapeutically equal to or better than D-tubocurarine, whilst others may find therapeutic applications of quite a different kind.

So far as chemotherapy is concerned, this has always been a primary interest in the organic chemical laboratories of the Institute. It cannot be denied that the workers in these laboratories have had their full share of the disappointments which seem to be inevitably associated with this most exacting and tantalizing form of scientific effort. Chemotherapy is a subject in which, at least until recent years, there has been little or no basis of theory upon which a logical attack of a problem can be planned. This being so, the probability of success has necessarily been determined by the quantitative magnitude rather than the qualitative nature of the experimental investigation. In comparison with the effort which has been put into chemotherapy by large industrial organizations, that at the Institute has, of course, been extremely small; nevertheless, it has not been without its successes, and in one case at least, namely, in the part played in the development of the amidine drugs for the treatment of kala azar, the practical outcome has been significant. At any rate there is every intention that chemotherapy in all its aspects shall continue to be a principal feature of research work at the Institute, particularly

in relation to the outstanding problems of the chemotherapeutic treatment of tuberculosis and of true virus diseases.

The staff of the chemical laboratory at the Institute have also been responsible for a major theoretical contribution to chemistry. This was the suggestion which was advanced in 1932 by Rosenheim and King for the structure of cholesterol and which brought about a reorientation of the chemistry of the whole group of steroids—a group that includes so many compounds of biological importance.

One of the more remarkable developments in medical and biological research in recent years is the increasingly important part which is played by physical methods. So far as the Institute is concerned the illustration of this, which is very much in my mind, is that the plans which had been made immediately before and during the early part of the recent war for the occupation of the new building have had to be fundamentally revised to provide the new facilities which are demanded by this particular branch of the work. We have already seen how even in the early stages of the virus work special physical and optical techniques had to be employed in order that the progress of the work should be maintained, but at this time the techniques in question were relatively simple, and modest in their demands for space and personal effort. Now it is entirely necessary to provide facilities for much more complicated procedures, such as phase contrast microscopy, electron microscopy, electrophoresis, high-speed centrifugation and ultrasonics. In addition to all this the introduction of the use of isotopes as valuable tools in biological research and particularly the almost unlimited availability of radioactive isotopes for this purpose has necessitated the undertaking of an amount of purely physical work which would have seemed fantastic for an institute of medical research even as little as 10 or 12 years ago.

All the physical techniques which I have mentioned are now available in the Institute, where they are used not only by workers within the Institute itself, but for those engaged in medical research problems in other institutions, who require to use special physical methods for their work but have not themselves access to the necessary apparatus and skilled help. This state of affairs is particularly noticeable in respect of the isotope work; on the one hand, the Institute mass spectrometer is made available for analyses for medical research workers in Medical Research Council Units or University laboratories who are using stable isotopes in their experiments; on the other, the staff of the Biophysics division have the task, at least so long as the present position of dependence on American supplies persists, of receiving, assaying and distributing the radioactive isotopes required for the many research projects which are being carried on throughout the country with the aid of these materials, in the medical and biological field. Naturally the ready availability of this variety of physical techniques within the Institute is itself a stimulus both to the further development of the methods themselves and to their application wherever this seems likely to aid the solution of a problem.

To return to the biological work of the Institute I must first refer to that of the Department of Applied Physiology. It will be remembered that this department, under the leadership of Leonard Hill, was one of the main divisions at the outset of the activities of the Institute, and it remained so until Hill's retirement in 1930.

The first main theme of work of this department was the study of environmental factors in their relation to health and activity, and among the more important results of this work was the development of the kata-thermometer, an instrument which has been widely used for many years and is still employed with good effect in the assessment of conditions of ventilation. Much time was also spent in the study of the physiological effects of ultra-violet light, and this work linked up with the inquiry already described which ultimately led to the isolation of calciferol. In still another aspect of its work the department was concerned with the physiological effects of high and low atmospheric pressures, the former particularly in relation to the problems of deep-sea diving. It is of some interest to note that after a lapse of 20 years there is once more to be a division of Applied Physiology within the Institute, the staff of which will again be engaged in the study of the effect of environmental conditions on various human activities.

Shortly after the department of applied physiology ceased to exist as a separate division the biological work of the Institute was extended in another direction by the establishment of a division of endocrinology under A. S. Parkes. This became a widely recognized centre for the study of the sex hormones in particular, and was responsible not only for advances in the knowledge of the fundamental physiology of these compounds, but for practical discoveries which facilitated their therapeutic use. The interest of the workers in this department in the anterior pituitary gland had also much to do with the development of the experiments of F. G. Young on diabetes which have already been mentioned. As time has gone on the activities of this division have extended beyond the limits of endocrinology, and its field of work is now better covered by the more general title of experimental biology, the current interest being chiefly in problems of fertility.

I come now to a part of the work of the Institute which is not confined to pure research, namely, the maintenance of biological standards. I have already referred to the work which had to be done during the 1914-18 war on the control of drugs, particularly arsenicals, and for which H. H. Dale was chiefly responsible. This work was the foundation of an enduring interest in problems of standardization of physiologically active compounds whose potency had to be assayed by biological means. Many substances which have important uses in therapeutics are not, particularly when they are first introduced, sufficiently well defined to be properly assayed by chemical and physical tests. Such a substance therefore has to be tested for potency by a biological test for its specific activity, and all biological tests are subject to an inherent variability which makes them unsuited in themselves as a basis of quantitative measurement. The only way out of the difficulty is to set up a permanent standard preparation of the substance in question and to define biological activity in terms of a known weight of this standard preparation. If new preparations are then tested biologically in direct comparison with the original standard or with a substandard prepared therefrom their activity can be expressed in terms of a known weight of a stable substance, instead of in terms of the behaviour of an animal on a particular day. It is on this fundamental principle that the whole work of biological standardization is based.

In the Report of the Council for 1920 we find the remark that at every point the

work of the departments at the National Institute for Medical Research has concern with biological standards, and it is noted that a department of biological standards had been set up at the Institute under H. H. Dale with appointment of an additional member of the staff for special work in connexion with it.

The main types of substances for which biological standards are needed are serological preparations such as antitoxins, vitamins, hormones, and certain drugs such as digitalis. In the early years at the Institute much work was done on the preparation of standards for diphtheria and tetanus antitoxins, digitalis, and posterior pituitary gland. In 1923 steps were taken to correlate the work which had been done up to that time on biological standards in this and other countries, and international agreement was obtained for the establishment of standards for diphtheria antitoxin and posterior pituitary gland. In the same year the League of Nations Health Organization assumed responsibility for biological standardization as a whole; the international status of the work has been maintained from that time; after the recent war responsibility was taken over by an interim commission and it is now passing into the hands of the World Health Organization. In 1922 began the long association with work on standards of Percival Hartley, who was appointed to the staff in that year and later directed the Department of Biological Standards within the Institute until 1946.

A further event which was of importance to medicine in this country occurred in 1925 when the Therapeutic Substances Act was passed. This Act provided for the official control of therapeutic substances whose potency had to be determined by biological means and in the exercise of this control a new responsibility was placed upon the staff of the Department of Biological Standards at Hampstead. In the schedules of the Act the units of activity of each of the therapeutic preparations are defined in terms of the standards which are preserved in the National Institute for Medical Research. The Department there is responsible for the issue of samples of the appropriate standards to approved manufacturers who may require them, and, furthermore, for ensuring that the products placed on the market by these manufacturers satisfy the requirements laid down in this Act.

As time has gone on and as new biological products have been brought into therapeutic use, the work of the Department of Biological Standards has considerably increased. There are now some thirty-seven standards preserved at Hampstead, of which thirty-five have international status, mostly in the groups of vitamins, hormones and serological preparations, with the addition recently of antibiotics such as penicillin and streptomycin. Samples of these standards are, as I have said, issued to manufacturers, who customarily prepare their own substandards for the control of their products, and with whom the closest touch is maintained on all technical questions arising from the work. The standards are also available to research workers in all countries. In many countries National Control Centres have been established, and where these exist distribution of the ultimate reference standards from Hampstead to the countries concerned is effected through them.

The actual way in which the work on standards is done at the Institute is perhaps worth noting. The amount of technical work required in connexion with the

establishment of new standards may be considerable, but the demands for such work are inevitably intermittent, and the experimental technique required may fall in any one of a large variety of fields. For this reason it has not been thought wise to establish a large department devoted exclusively to work on standards. The director of the Department has the responsibility for the co-ordination of the whole of the work, and for expert advice and assistance he is able to call on his colleagues in the other departments of the Institute who possess the relevant special knowledge. This arrangement has the great advantage that the necessary technical work becomes an occasional demand on the time of members of the scientific staff who are otherwise engaged on their own research problems, instead of being a full-time occupation of a somewhat restrictive character. In actual fact many members of the staff who have given most help with standards, including those in the Department itself, have themselves been responsible for some of the more important contributions in research. One need only recall the researches of Hartley himself on general problems of immunology and those of Bruce White on the cholera vibrio to realize the truth of this statement, whilst among workers less closely connected with the Department, the physiologists and the endocrinologists have given much valuable assistance to this work.

Biological standards, besides forming an essential part of the system of control of biological therapeutic products, offer a substantial service to research; moreover, the work which arises in connexion with them itself stimulates research particularly in the important field of biological assay. The existence of the Department is furthermore of great value to the Institute itself; through the department valuable contacts are maintained with the research laboratories of industrial firms, and, since the preparatory work for the establishment of new standards is always done so far as possible with international co-operation, contacts are also close with research workers in other countries.

There is no part of a research institute which is of more importance to the general progress of the work than the Library; I cannot conclude my description of the National Institute for Medical Research without mentioning that as a result of the liberal policy of the Council and of the devoted efforts of the librarians the Institute now possesses a library which, having started from very small beginnings, has become one of the best medical research libraries in the country. The facilities of this library are of course not restricted to workers within the Institute itself, but are available to other workers under the Council, and, by special arrangement, to medical research workers elsewhere.

This brings me to the end of my outline of the main activities of the National Institute for Medical Research. It has been an account which makes no claim to completeness, but in which selected themes have been briefly discussed as illustrations of the type of work which is done and of the main trends of development. The story which I have told is one of an expanding effort, the expansion relating both to the numbers of the scientific staff and to the range of scientific work which is covered. This expansion has been continuous, not even being completely interrupted by the recent war. During the war the activities of the Institute were fully maintained, although there was inevitably and properly a considerable temporary

redirection of the research effort to problems of immediate importance; among these may be mentioned in passing the work of the physiologists on underwater physiology in connexion with submarine warfare, work on measures of protection of troops against flamethrower attack, and studies of protective measures against rickettsial diseases of military importance. With all this it was possible to keep pace with new technical developments and to maintain a background of fundamental research so that resumption of normal activities on an increased scale was not too long delayed.

Looking in more detail at the process of expansion of the work at the Institute from the early days of the three laboratory departments to the present very much larger and more complex establishment we see an increasing emphasis on biochemistry and biophysics on the one hand together with a broadening of the biological research through endocrinology to more general aspects of experimental biology on the other. In these respects the development is a true reflexion of the general trend during the past thirty years of medical research in the laboratory, which must make ever wider demands on the whole field of scientific effort if it is to progress. The very increase in the range of work which has to be covered, with its accompaniment of multiplication of departments with differing immediate interests, adds weight to the principle laid down by the Council in the early days of the Institute that there must be the freest contact between the different laboratories.

If the organization which I have depicted seems to some to be ill-defined and loose in structure, this is because of the pre-eminent importance which is attached to the principle of flexibility and avoidance of departmentalization. It is, indeed, in my view only with the strictest adherence to such a principle, and within the framework of the early declaration of research policy which I quoted at the beginning of this lecture, that we can hope for the Institute to continue to fulfil its proper function of producing new knowledge in science and medicine, and in doing so to live up to the achievements of the past, of which it may reasonably be proud.

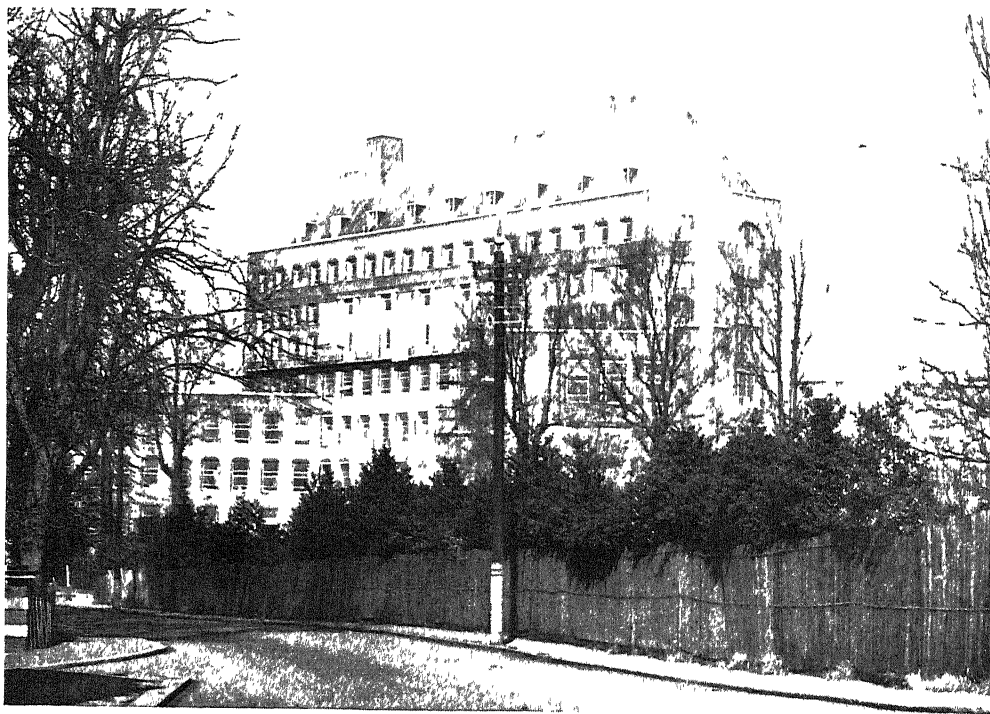




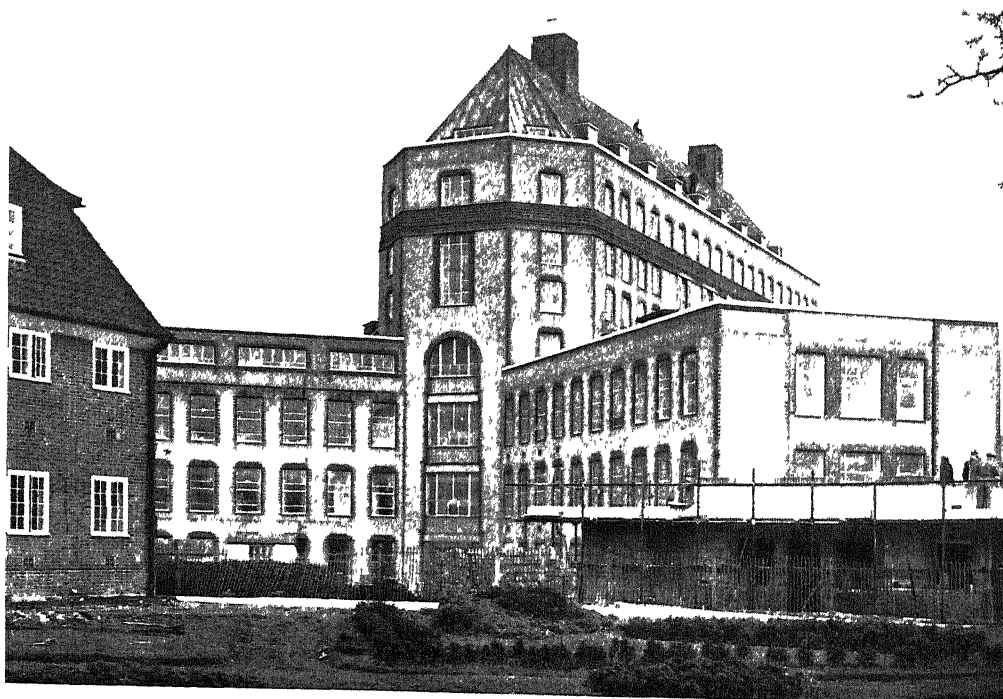
The National Institute for Medical Research, Hampstead View of front of building.



The National Institute for Medical Research, Hampstead View of back of  
building showing Ronan Extension at left-hand side (*facing p 348*)



New National Institute for Medical Research, Mill Hill (In course of completion)  
View from the south east



Western end of new National Institute for Medical Research, Mill Hill (In course of completion.)

# The Royal Greenwich Observatory

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(*Lecture delivered 27 January 1949. Received 27 January 1949*)

[Plates 15 to 18]

The Royal Observatory was established by King Charles II in the year 1675 for the specific practical purpose of 'rectifying the tables of the motions of the heavens, and the places of the fixed stars, so as to find out the so-much-desired longitude of places for the perfecting the art of navigation'. At that time the most accurate star catalogue available was the catalogue of 1000 stars, prepared by Tycho Brahe about 1598; only 777 of the stars had been properly observed, and the star places, whose average errors were of the order of 1' to 2', were not sufficiently accurate for the purpose of determining longitudes. The best tables for giving the position of the Moon were liable to errors as great as 20'. Flamsteed, who was appointed the first Astronomer Royal, realized that a good stock of observations, continued for many years, was needed in order to provide star places with all the accuracy that was attainable and to furnish positions of the Sun, Moon and planets which could serve as the basis for the construction of satisfactory tables of their motions. So, from its very foundation, the Observatory started upon systematic and long-continued programmes of observation which, throughout its history, have formed its most significant and important contribution to astronomy.

## MERIDIAN ASTRONOMY

Until after the middle of the eighteenth century, when William Herschel embarked upon his studies of the structure of the sidereal system and upon his observations of star clusters, nebulae and double stars, astronomical observation was concerned almost entirely with the positions and motions of the heavenly bodies. But long after Herschel's pioneer work had opened up new fields of investigation, the observations at Greenwich continued to follow closely the lines originally laid down. There were improvements, of course, in the design and construction of instruments and in their optical quality; there were progressive refinements in technique and progressive improvements in accuracy, which in turn opened up new fields of investigation. The observations which Bradley made at Greenwich between 1750 and 1762, amounting to about 60,000 in all, were of a higher accuracy than any made previously and are, in fact, the earliest observations which are precise enough to be of use to the astronomers of to-day. Bradley was particularly careful in examining the errors of his instruments and in keeping the instruments in the best adjustment; he was the first to introduce corrections to atmospheric refraction for the temperature of the air and for the height of the barometer.

Halley in 1718 called attention to the fact that three of the bright stars, Sirius, Procyon and Arcturus, had changed their positions since Greek times, and that Sirius had perceptibly changed its position since the time of Tycho Brahe; the fixed stars were not, in fact, fixed, and the study of their proper motions added a new interest to the determination of stellar positions. Bradley himself discovered the two phenomena of stellar aberration and of nutation. William Herschel, by analyzing the proper motions of fourteen stars which had been determined with accuracy by Maskelyne at Greenwich was able to show that the Sun itself had a motion relative to the stars. The improvements in the tables of the motions of the Sun, Moon and planets, which resulted from the progressive increase in accuracy of observation and from the fact that observations were made all round their orbits, enabled the great Continental mathematicians of the eighteenth century, Euler, Clairaut, D'Alembert, Lagrange and Laplace, to prove that within the limits of accuracy of observation the movements of the bodies in the solar system could be accounted for in detail on the sole hypothesis of the Newtonian theory of gravitation.

These facts are mentioned to emphasize that the determination of the positions and motions of the Sun, Moon, planets and stars is work of fundamental importance in astronomy. It demands continuity of observations over a long period, made with the greatest care and precision. It was the work for which the Observatory was founded; it is the work which must always be its first concern, for it is work that is never completed but that will always go on. In 1875 Airy, after reviewing the work of 40 years at Greenwich since his appointment as Astronomer Royal, in the course of which he had expanded the work into various new directions, remarked:

'Turning now from the past to the future, I see little in which I could suggest any change. If it should ever be necessary to make any reduction, I should propose to withdraw Meteorology, Photoheliography, and Spectroscopy; not as unimportant in themselves, or as ill-fitted to the discipline of the Observatory, but as the least connected with the fundamental idea of our Establishment.'

Until well into the nineteenth century, meridian telescopes for the determination of position formed essential equipment of most observatories. Many amateurs even made regular meridian observations and provided significant contributions to positional astronomy. But as new fields of investigation in astronomy have been developed, meridian observations have been discontinued at most observatories. They make heavy demands on observing resources and involve much computational work. For this branch of astronomy, continuity of observations with the same instruments over a long period is of the greatest value. Such observations are therefore not well fitted for University or private observatories. It is proper that they should be undertaken almost entirely, as in fact they are to-day, by the national observatories. In recent years the systematic pursuit of meridian observations has tended to be restricted more and more in the northern hemisphere to three observatories, Greenwich, Washington and Pulkowa. During the war the Pulkowa Observatory was completely destroyed, though it is now being rebuilt on a larger scale, and meridian observations will continue to form an important portion of its work. At the end of 1940 meridian observations at Greenwich had to be dis-

continued for the first time in the history of the Observatory; the complete discontinuance was not for long, but until after the end of the war it was possible to carry on the observations only on a small scale.

The purpose of meridian astronomy is to provide a fundamental system of reference, defined by the positions and proper motions of a network of stars distributed with reasonable uniformity over the whole sky, together with the numerical value of the constant of precession. The observations are made from a moving and rotating earth and it is convenient to use the equator as a plane of reference and the equinox as a zero point. Both the equator and the equinox depend upon the motion of the Earth and the motion of its axis; they can therefore be determined by observations of the Sun which, in effect, fix the orbit of the Earth, or by observations of the bright inner planets, which involve the elements both of the orbit of the planet and of the orbit of the Earth. Observations of the Sun are peculiarly liable to errors of a systematic nature, while observations of the inner planets, showing perceptible disks and phases, are liable to personal errors which are different from those that affect star observations. There is the further difference that observations of the Sun and planets are made by day, while the star observations are mostly made at night.

The position of the pole can be determined from observations of circumpolar stars, and the position of the nadir can be determined from observations with a mercury horizon. The two together will fix the equator point. There are normally systematic differences between the equator point fixed in this way and the equator point deduced from Sun and planet observations. The circumpolar observations, above and below pole, are made 12 hr. apart; they may be affected by systematic diurnal effects, by errors in the corrections applied for refraction, by changes in the instrumental adjustments, by instrumental flexure, which is different for the two observations, and by errors in the adopted figure of the instrumental pivots. There are consequently many possibilities of errors of a systematic or quasi-systematic nature. When observations of stars in the equatorial belt made at a northern and at a southern observatory are compared, systematic differences are generally found. The observations can be brought into agreement by adjusting the coefficients of refraction used in the reductions of the observations at the two observatories, but the corrections so obtained are usually found to be quite inadmissible.

The observations made with the conventional type of transit circle are, in fact, liable to many sources of error which are difficult to control adequately. If the same stars are observed with two different transit circles and the derived positions are compared, it is found that, in addition to random errors of observation, there are systematic errors in both right ascension and declinations which vary with right ascension and also with declination. Magnitude error in right ascension, which affected observations made by the old methods of eye and ear and of hand-tapping, have been practically eliminated by the use of the impersonal micrometer.

The sources of error with the conventional transit circle are numerous. The errors of instrumental adjustment—of level, of azimuth, and of collimation—are continually varying. The variations are difficult to control adequately. If the meridian opening in the transit pavilion is narrow, refraction anomalies are inevitable; with

a wide opening, the instrument is fully exposed to the wind and to temperature changes. The errors of azimuth are particularly difficult to control, unless the site is one which permits of fixed azimuth marks of high stability. Corrections must be applied for any departure of the figures of the pivots from perfect cylindricality; the determination of the figures of the pivots with accuracy and with freedom from spurious elliptical terms is not easy. Instrumental flexure can be determined only in the horizontal position of the telescope, so that a law of variation with zenith distance must be assumed. The telescope is turned in the course of observation into all sorts of positions, with possibilities of displacements of objective or of the mechanical parts of the micrometer, which may be sufficiently small to escape easy detection but which can introduce serious errors of a systematic nature.

The difficulty of eliminating systematic errors of instrumental origin can be illustrated by the reversible transit circle of the Cape Observatory, which was designed by Sir David Gill with much thought and care. The telescope can be reversed in its bearings, giving two positions for observation, denoted by E and W; the object glass and eye-end are arranged to be interchangeable, giving two arrangements denoted by I and II. Four separate combinations are therefore possible. After allowing for the difference in horizontal flexure in the two conditions I and II, the difference in the declinations measured in the two conditions has a regular run amounting to  $0''.4$  from dec.  $+40^\circ$  to the south pole. The difference between the two positions E and W in both conditions has a range of  $0''.18$ , but the difference is systematically larger in condition II than in condition I, the extreme differences being  $0''.3$  and  $0''.2$  respectively. The mean of the observations in the four separate combinations is adopted as likely to provide the closest approximation to the truth.

Because of these systematic errors of instrumental origin, the proper motions of stars must be based largely upon series of catalogues observed over many years with the same instrument, whereby the instrumental peculiarities are to a large extent eliminated. The observations made at Greenwich are of special importance for this purpose, as the Airy transit circle, which defines the prime meridian of longitude, has been used continuously since 1851. With this instrument more than 650,000 observations have been made. The last three programmes consisted of the observations of all the stars down to about the 8th magnitude in the zones of declination  $0^\circ$  to  $+32^\circ$ ,  $+32^\circ$  to  $+64^\circ$ ,  $+64^\circ$  to  $+90^\circ$ , supplemented by fainter stars in regions of high galactic latitude, together with numerous observations of a large number of fundamental stars. Thus the whole of the northern sky has been covered. The proper motions of the stars have also been investigated. The Airy instrument does not accord with modern ideas for the construction of a transit circle; it is not reversible in its bearings; it is housed in a pavilion which has buildings on each side with a narrow opening and liability to refraction anomalies; the terrain is asymmetrical to north and south, with the possibility that the atmospheric refractions north and south of the zenith may not be equal. The instrument has now reached the end of its useful life. A new transit circle, reversible and housed in a pavilion of semi-cylindrical shape, with a wide aperture and a fairly symmetrical terrain to north and south, was installed shortly before the war. Before bringing this instru-

ment into regular use, it has been subjected to a lengthy series of investigations, in the course of which the errors of the circle graduations and of the figures of the pivots have been determined with great accuracy. These investigations have thrown much light upon various ways in which the observations can be affected by systematic or quasi-systematic errors of instrumental origin.

These remarks have been made to illustrate the difficulties and complexities of meridian astronomy and to explain why observations of position must still be continued. The observations made at different times and with different instruments at different observatories are combined, by a process of adjustment which is to a large extent empirical, to form what is termed a fundamental system, so providing a system of reference that is more accurate than it is possible to obtain from observations with a single instrument and, moreover, covering the whole sky. The more effectively the various sources of error can be eliminated or controlled with each particular instrument, the more reliable the fundamental system will be. Both the places of the stars and their proper motions, as given in the fundamental system, are affected by errors; the errors of the star places increase with the lapse of time from the epoch of the system, so that successive revisions of the system become necessary. A process of gradual approximation, which still continues, is involved.

With the realization of the serious difficulties involved in the use of a large movable telescope for the accurate determination of positions, it is natural that consideration should be given to alternative designs of instruments in which the movable parts are reduced as much as possible. The use of a fixed telescope in conjunction with a moving plane mirror has more than once been suggested; a design of such a mirror transit circle has been developed at Greenwich. It is proposed to employ two fixed horizontal telescopes, with their axes in the meridian and their objectives facing a movable mirror, whose plane is parallel to the east-west axis of rotation. Stars would be observed in one or the other telescope according to whether they transit north or south of the meridian, while the two telescopes would serve also as collimators. Such an arrangement reduces moving parts to a minimum. The fixed telescopes can be of longer focal length than is convenient for a movable telescope, and they can be effectively insulated against rapid temperature changes. Observations of nadir, of level and of near-zenith stars can be taken in either telescope. Flexure effects are reduced to a minimum, while troubles arising from small displacements of micrometer parts or of objectives are entirely eliminated. The adaptation of a variable-speed motor drive to the micrometer wire is simplified. A small model of the proposed design has been constructed and the theory of the instrument has been investigated. The instrument appears to have great possibilities and it is hoped to try it out in practice.

Closely related to the meridian astronomy is the determination of the variation of latitude caused by the movement of the Earth's axis of rotation relative to the Earth. The pole has an irregular motion within a circle of about 30 ft. radius; it contains two principal components, of periods 12 and 14 months, but the motion is not sufficiently regular to predict ahead. The component of the motion along the meridian of a place causes a change of latitude; the component in the perpendicular direction affects time determinations. The complete motions can be determined by



observations of the variations of latitude at two places whose longitudes differ by about  $90^\circ$ . The variation of latitude was first established in 1888; Chandler afterwards found it clearly exhibited in observations back to 1750. Determinations were first made at Greenwich with the Airy reflex zenith tube, but the observations were not entirely satisfactory. A long series of observations was commenced in 1911 with the Cookson floating zenith telescope, the observations being made photographically. Pairs of stars at nearly equal distance north and south of the zenith are observed at meridian passage; each star makes a trail across the plate, the telescope being rotated through  $180^\circ$  between the observations of the two stars. The separations of the pairs of trails are measured, and the variations in latitude are deduced from the changes in these separations. Meridian observations of declination are corrected for the variation of latitude. A new photographic zenith tube, now under construction, will be used, when completed, for the determination of the variation of latitude as well as for time determination. The study of the polar motions raises many interesting problems and provides a means for the determination both of the constant of nutation and of the constant of aberration.

#### TIME DEPARTMENT

The provision of a time service is a normal function of a national observatory and is closely related to the work of the meridian department. The right ascension of a star is the sidereal time of meridian transit of the star. In positional astronomy a selected number of the brighter stars in the equatorial belt, suitably distributed round the sky, are selected as 'clock stars'. The positions and proper motions of the clock stars, as used at Greenwich, have been derived from the long series of meridian observations and are progressively refined as the observations continue. Observations extending from 6 to 12 hr. serve to control periodic errors in the right ascensions of the clock stars. Using these adopted right ascensions, the observations of the clock stars determine the errors of the standard sidereal clock night by night. The right ascension of any other star is derived by correcting the observed sidereal time of its transit for the clock error, interpolated for the time of transit.

Before 1927 the time determined at the Royal Observatory was based upon observations with the Airy transit circle. In 1926 a world programme of longitude determinations, in which a large number of observatories in all parts of the world participated, was undertaken under the auspices of the International Astronomical Union. For this special programme, a small reversible transit circle was used at Greenwich, the telescope being reversed near the middle of each transit, thereby eliminating the correction for collimation error. For such a programme it is necessary to adopt a common system of star places, in order to ensure that the derived longitudes are as free as possible from systematic errors in star positions determined with different instruments; in this particular programme the star places in Eichelberger's *Fundamental Catalogue* were used. It was found that the clock errors derived from these observations had a much smoother run than the clock errors derived concurrently from observations with the Airy transit circle. The latter are affected by obscure instrumental errors, to which reference has already



been made. It was found, moreover, that when the smoother clock errors provided by the small transit observations were used for the reduction of the Airy transit circle observations, the derived right ascensions of the stars became discordant; but that when the more irregular errors given by the transit circle observations were used, the derived right ascensions became accordant. The instrumental peculiarities of the transit circle are evidently involved.

Since 1927, therefore, the time determinations have been based entirely upon observations with small transit instruments, which are reversed in the middle of each transit. By resolution of the International Astronomical Union the revised Auwers fundamental system, known as the FK 3, is used for the system of star places. The apparent places of the stars are taken from the annual volume *Apparent Places of Fundamental Stars*, published by the Nautical Almanac Office.

Prior to 1923 the standard clocks employed in the Royal Observatory were regulator clocks with Graham dead-beat escapements. A Cottingham clock, fitted with a Riefler escapement, installed after the first World War, was expected to give a higher standard of performance, but failed to come up to expectations. The development of the Shortt free-pendulum clock introduced a new standard of precision in time-keeping. In this type of clock a master pendulum, mounted in an airtight case, exhausted to a pressure of about 1 in. of mercury, and placed in a constant-temperature room, synchronizes a slave clock of commercial type. The master pendulum swings freely, except when given a small impulse once each half-minute, and is relieved of the work of moving a train of wheels to show the time on dials and of sending out signals. The first clock of this type, Shortt no. 3, was installed at the Observatory in November 1924 and soon showed its superiority over other types of pendulum clock. Other clocks of this type were therefore installed and used both as sidereal and as mean time standards in the Observatory.

The introduction of the new standards resulted in an important change in the system of time employed. The transit of the first point of Aries or the vernal equinox defines the beginning of the sidereal day, 0 hr. sidereal time. But the precessional motion of the true equinox is not uniform, being affected by irregularities, due to solar and lunar perturbations, which are known as *nutation*. In consequence the sidereal day varies slightly in length. If we imagine a point moving uniformly along the equator, with a motion equal to the mean motion of the true equinox, and so that its extreme distances from the true equinox on both sides are equal, we may term this point the *mean equinox*. The sidereal time determined by observation is *apparent sidereal time*. A *mean sidereal time* can be defined by reference to the mean equinox, in which all days are of equal length; it is obtained by subtracting the nutation from the apparent sidereal time. Apparent sidereal time was good enough before the introduction of the Shortt free-pendulum clocks; their superior precision made it necessary to introduce the concept of mean sidereal time, which has been universally adopted. The detailed study of the performance of the Shortt clocks at Greenwich, which proved their superiority over other types of pendulum clocks, stimulated their introduction into observatories in many parts of the world.

The Royal Observatory is responsible for the distribution of time to the public. The first steps in the distribution outside the Observatory became possible with the development of telegraphic communications. In 1852 an electric clock was installed at the Observatory, which transmitted a signal each day that caused a time-ball, on the offices of the Electric Telegraph Company in the Strand, to drop. In 1865 signals were sent hourly to the Electric and International Telegraph Company's office, whence they were distributed over the railway network of the country. After the telegraph system was taken over by the Post Office, in 1870, a complete system for the hourly distribution of time through the Post Office was gradually developed, which made Greenwich time widely available. A further step in the widespread dissemination of accurate time followed naturally upon the development of broadcasting. Two of the Dent regulator clocks were modified to run as synchronized clocks under the control of one of the mean time free pendulums, and were provided with a system of contacts to enable time signals to be transmitted automatically every quarter of an hour to the British Broadcasting Corporation. The signals were in the form of six dots at intervals of a second, the last coming exactly at the hour, the quarter, or the half hour. These signals are the familiar B.B.C. 'six pips' Greenwich time signal, which, since 5 February 1924, have been transmitted on all B.B.C. wave-lengths several times daily.

A further service, designed to be of value for navigation, was commenced on 19 December 1927. From that date radio time signals have been sent out on a frequency of 16 kc./sec. twice daily, at 10 and 18 hr. G.M.T., from the Observatory, via the Rugby wireless station. These signals, which last for 5 min., are of the so-called vernier type, spaced 61 to the minute, enabling the error of a chronometer to be accurately determined by observing the instants of coincidence between the signals and the ticks of the chronometer. Corrections to the times at which the signals were emitted are published by the Observatory at approximately monthly intervals, for use where higher precision is required, as, for instance, in survey operations. For the distribution of these signals a special 'diminished seconds' slave clock was installed, whose pendulum swings 61 times in a minute, and which is kept in synchronization by the mean-time master pendulum. The service has more recently been extended by simultaneous transmission of the time signals on several short wave-lengths. The introduction in 1936 by the Post Office of the 'speaking clock', designed by the Post Office engineers and constructed at the Post Office Research Station, which is automatically controlled by hourly time signals from the Observatory, has made accurate time continuously available.

It is necessary that the Observatory should keep abreast of developments in precision horology so that the time service can meet all demands for precision that are made upon it. After the development of the quartz crystal clock, it soon became evident that a new standard of precision in time-keeping had been reached. It was therefore decided to instal a clock of this type at the Observatory in order that some direct experience could be gained of its performance in comparison with the performance of the free-pendulum clocks. A clock, using a Dye-Essen ring crystal, was constructed under the supervision of the National Physical Laboratory and installed in 1939. The quartz vibrator was adjusted to have a frequency of 100 kc.

per sidereal second; demultiplier circuits provided an output with a frequency of 500 cycles per sidereal second, which was used to drive a phonic motor. Although the performance of this clock was not altogether satisfactory, experience showed that it was free from the small erratic changes of rate to which the pendulum clocks were liable and which made prediction uncertain; it was found also that it would be more convenient, when additional quartz clocks were installed, to employ a fundamental frequency of 100 kc. per mean solar second instead of per mean sidereal second.

The outbreak of war stopped for a time developments which had been planned for an improved time service. A skeleton time service was at once installed at the Abinger Magnetic Station as a safeguard against the possibility of the service from Greenwich being put out of action. Towards the end of 1940 when the frequent air raids made night observations impossible at Greenwich, the time service was moved in its entirety from Greenwich to Abinger. Shortly afterwards a second time service was installed at the Royal Observatory, Edinburgh, with the co-operation of the Astronomer Royal for Scotland. For the remainder of the war, the two time stations, at Abinger and Edinburgh, were in use; the two stations were connected by direct land line with teleprinter communication, enabling clocks at either station to be recorded at the other station.

Plans were proceeded with for the installation of quartz-controlled frequency standards. The first group of three clocks was installed in 1943. These clocks, which were constructed at the Post Office Research Station, were of the Post Office Group IV type, in which a GT-cut plate of quartz is maintained in oscillation at 100 kc. per mean time second, by a bridge drive circuit, which reduces to a minimum the effect of variation in the supply voltages. The crystals are mounted in thermostatically controlled ovens, and the temperature range permitted by the modified Turner circuit employed ensures that variations in frequency from this cause are small. Regenerative frequency dividers are used to provide an output at 1000 c./sec., which can operate phonic motors, provided with contacts from which signals can be taken off.

The installation has since been considerably extended and the time service is now based upon six groups of three quartz crystal clocks, four of the groups being at Abinger, where the main time-service station has remained, and two at Greenwich, enabling a time service of much higher precision to be provided than was possible with pendulum clocks. The quartz clocks have the further advantage that relative errors and rates can be obtained much more readily and with much greater accuracy than with pendulum clocks. For measuring time and frequency differences, decimal counter chronometers are used, embodying scale-of-ten counter circuits. The 100 kc./sec. output from one of the primary standards is fed into the counting unit. A seconds impulse from one clock can be applied to start the count and a seconds impulse from another clock to stop it. A single reading of the time difference between the two clocks is obtained, being shown on five dials reading successively in units of tenths, hundredths, thousandths, ten-thousandths, and hundred-thousandths of a second. To provide a check, the roles of the two clocks can be interchanged. Alternatively, by throwing over a switch, a series of successive readings may be obtained, when the results are added in the counter. This procedure is of value when

the intervals are nominally constant but are subject to small erratic variations, as when comparing a clock with a time signal; ten consecutive readings can then be obtained with advantage. For frequency comparisons, the nominal 100 kc./sec. outputs from two clocks are fed into a comparator, where the levels are adjusted to a standard value. The adjusted outputs are then combined and the resultant beats are shown on a meter. By means of a trigger circuit, a pulse is sent to the decimal counter when the beat-frequency voltage passes through zero. The intervals between the beats are thus accurately timed, enabling frequency differences to be determined with an accuracy of at least one part in  $10^{10}$ . The quartz clocks are provided in addition with automatic beat counters; by automatic counting of the number of beats between each pair of oscillators in a 24 hr. period, the change in the time difference between each pair of clocks in the course of a day is recorded in units of  $10^{-5}$  sec. The intercomparisons between each pair of clocks, in each group of three, provides an automatic check against incorrect action of any of the beat counters.

An additional advantage of the quartz crystal clocks is that though they are rated approximately to mean time they can serve all purposes. Separate sidereal time clocks and diminished seconds transmitters for the rhythmic time signals are no longer required. The phonic motors, driven by the 1000 c./sec. output from one of the clocks, can be adapted to these additional requirements. In the phonic motors used at the Royal Observatory, the rotor consists of a laminated iron ring with 100 teeth cut on its inside surface. The six-pole stator within the rotor has corresponding teeth cut on its pole faces. The motor runs synchronously at 10 revolutions per second, driving by gearing a commutator wheel at one revolution per second. A contact spring, bearing on this wheel, closes an electrical circuit for one-tenth of each second. In order to obtain sidereal seconds, a special gearing is used. The gearing ratio employed is  $\frac{119}{114} \times \frac{317}{330}$ , which is about four parts in  $10^9$  smaller than the correct ratio. This small error in the gear ratio is immaterial, for when the rate of the clock in mean-time milliseconds has been determined, the rate of the sidereal impulses, in sidereal-time milliseconds, can be at once inferred.

The rhythmic time signals consist of a long dash at the minute, followed by a series of dots spaced at intervals of  $\frac{1}{61}$  min. These signals are derived from a contact drum, which is driven through a 60/61 gear from a phonic motor. In order that the signals may be sent out at the desired instants, a phasing adjustment is provided for the signal transmitter. There are day-to-day variations in the time lag introduced by the land-line joining the Observatory to the Rugby wireless station. Some test signals are transmitted a few minutes before the time signals themselves; these test signals are received and recorded at the Observatory and by comparing the times with those of the outgoing signals, the land-line lag is deduced. An adjustment to correct for the lag is made by rotating the phonic motor stator, thus advancing or retarding the phase of the rotor. A rotation of the stator housing by  $360^\circ$  advances or retards the contact time by one-tenth of a second.

The most recent phonic motor equipment installed at the Observatory provides a complete contact assembly for the control of all the time signals sent out, including

not only the rhythmic signals but also the B.B.C. 'six pips' time signals and the hourly signals for the control of the Post Office speaking clock.

The use of quartz clocks has resulted in a much improved precision in the time service. Their freedom from small erratic changes of rate makes accurate short-term prediction possible. This is of importance for the control of precision frequency standards, which can be checked against a 24 hr. time interval of high precision. The Rugby 10 hr. time signals are transmitted so as to give an accuracy in the 24 hr. interval between the signals on consecutive days which does not normally exceed 1 msec. The difficulties of accurate long-term prediction, which is needed to carry over periods during which no time determinations can be obtained, are much greater. The quartz crystal oscillators are subject to an ageing effect, which causes a drift in frequency, more rapid at first and gradually decreasing, though never, as far as present experience is a guide, completely disappearing. Time determinations extending over some months are needed in order to derive the frequency drift with the accuracy needed for prediction. But a complication is introduced by the motion of the Earth's poles, which causes small displacements of the meridian; the result is that a perfect clock, compared with absolutely accurate time determinations, would appear to have a small variable error, which at Greenwich can amount to about  $\pm 25$  msec. As the polar motion has two principal components, with periods of 12 and 14 months, an incorrect determination of frequency drift is inevitable unless the effects of polar motion can be allowed for. The motion of the pole along the meridian can be determined by observing the changes of latitude which result from it; the motion in the perpendicular direction can be determined only from observations of latitude variation at another observatory, differing by about  $90^\circ$  in longitude. The determinations of latitude variation at Washington, longitude  $77^\circ$  W, are communicated regularly to Greenwich and are used to correct for the effects of polar motion and thereby to derive more accurate values of the frequency drifts of the clocks. It is noticeable that the application of these corrections has appreciably smoothed the apparent errors of the clocks.

The introduction of quartz crystal clocks has demanded an improvement in the precision of the time determinations. The period of several months, which is required for a satisfactory determination of frequency drift with the relatively large errors inherent in the time determinations with the small transit instruments, could be much reduced if an appreciable reduction in the errors of observation were achieved. Much consideration has therefore been given in recent years to the design of new instruments for time determinations.

A photographic zenith tube, which is expected to reduce the probable error of a time determination to a few milliseconds, is now in an advanced stage of construction. This instrument is based on Airy's design of the reflex zenith tube at Greenwich, with modifications to adapt it for photographic observation due to F. E. Ross and incorporated in his photographic zenith tube, now in Washington. The essential principle is the employment of a zenith telescope, whose tube contains a mercury horizon to reflect the light and to bring it to a focus in the second Gaussian point of the objective, thereby making the observations practically independent of any error of level. The important modification introduced by Ross

was the inversion of the objective, placing the flint component uppermost, whereby, with an appropriate separation between the two components, the second Gaussian point is brought a few millimetres below the lower face of the crown component. The observations are made photographically, the photographic plate being mounted in the Gaussian plane. The instrument was designed originally for the measurement of the variation of latitude; the upper portion of the instrument, which carries the objective and the photographic plate, is in the form of a rotary, which can be turned through exactly  $180^\circ$ . If two exposures are made on a star, the rotary being turned through  $180^\circ$  between them, the separation between the two images in the direction of the meridian is twice the zenith distance of the star. In practice, exposures of finite length are given, the plate carriage being travelled along during each exposure with the speed of motion of the star image. If the two exposures are accurately timed and are approximately symmetrical about the instant of meridian transit, the time of transit can be inferred from the small relative displacement of the images in the direction perpendicular to the meridian.

The advantages of this type of instrument for time determination are considerable. The observations being photographic, personal equations are eliminated. Errors of level do not affect the observations; there is no collimation correction to trouble about; observations in the zenith are independent of azimuth error. As the instrument is fixed, the various sources of error to which a moving instrument is liable cannot occur. A longer focal length can be used than is possible with a moving instrument, with the advantage of a correspondingly greater scale. Observations are restricted to the zenith, where atmospheric transparency is highest and refraction effects are at a minimum.

The instrument which has been designed at Greenwich differs in a number of important respects from the Washington instrument:

- (i) It has a larger aperture (10 in.) and longer focal length (135 in.).
- (ii) A plain ball-bearing is used for constraint of the rotary, in place of conical bearing, in order to reduce friction and to facilitate construction.
- (iii) An autocollimation method is used as a criterion of the angle of reversal of the rotary.
- (iv) As a fixed axis of rotation is not required for (iii), a definite constraint in the horizontal plane is not needed. The two working orientations are each defined by a pair of stops instead of by a single stop.
- (v) Adjustments to the objective are provided for squaring-on and for coincidence of the nodal plane and photographic plate.
- (vi) Automatic reversal is accomplished by means of a system of wires which exert a pure torque on the rotary and therefore no tilting torque on the tube. The system is such that unidirectional rotation of the driving shaft is converted into reciprocating rotation of the rotary.
- (vii) The plate carriage is annular and the plate-holder mount is circular so that symmetry of diffraction pattern is secured. The carriage constraints are external to the aperture.
- (viii) Relative motion of the carriage and rotary is made to approximate to pure translation by means of a compensating system of flexed rods, which constrain the

carriage in the horizontal plane to which the motion is restricted by means of three balls that roll between horizontal planes.

(ix) Uniformity of rate in the relative translation of carriage and rotary is obtained by a specially designed system comprising a differential roller and metallic tapes.

(x) The time scale is produced photographically by means of a clock-controlled lamp giving flashes of very short duration. An independent chronograph is not required.

(xi) The height of the mercury surface is accurately adjustable and, as criterion of adjustment for constancy of scale value, an optical null method has been introduced for use in conjunction with a suspended silica rod.

Some consideration has also been given to the design of a new type of transit instrument, designated as the Horizontal Transit Instrument. The essential feature is that the telescope system remains fixed (though adjustable) with its axis horizontal and in an east-west direction. The light from a star of any declination, near the position of meridian transit, is directed along the optical axis by a subsidiary optical system of constant deviation, which can be rotated about an east-west axis and can be set to the appropriate declination. The effect on time determination of its positional errors (whether due to maladjustment of the axis of rotation or to pivotal errors) is reduced to the second order. Level and azimuth errors of the telescopic system have the same effect on the observed time of transit as they do with the ordinary transit instrument; but since the telescopic system is not deliberately subjected to gross mechanical disturbances and suffers from no pivotal errors, these level and azimuth errors should be far more stable than in the reversible instrument. The collimation error is dealt with by duplication of the telescopic system and reversal of the subsidiary system, so that the essential advantage of the reversible instrument is not sacrificed. Observation is made at the common focal plane of the duplex telescopic system, from the two sides successively. The fixity of the telescopic system avoids errors due to flexure, and permits of the use of a focal length considerably greater than can profitably be used in the ordinary reversible instrument. The level is determined with reference to two mercury surfaces, one at each end of the instrument, by means of an autocollimation method.

Instead of following the star image with a movable micrometer wire, a variable-deviation system is used by which the light in the telescopic portion of the instrument is kept always axial as the direction of the incident starlight rotates. In this way the tolerances of certain essential adjustments are greatly increased. Further, this variable-deviation system acts also as a micrometer and as the means by which signals are sent to the chronograph. An additional advantage of this axial method is that the fiducial line that bisects the star image is not required to move in order to follow the star's image or to be linked to the signalling system as at present. Thus no mechanical errors are introduced at this point. An optical method is contemplated for defining the position of the variable-deviation system in such a way that in its performance as a micrometer or signal emitter the system will be effectively free from the effects of mechanical errors. A thorough examination of the theoretical aspects of the design has been completed.

## MAGNETIC AND METEOROLOGICAL DEPARTMENT

The first extension of the work of the Observatory beyond that laid down in the Royal Warrant for its foundation came with the setting up by Airy in 1840 of a magnetic and meteorological department. Certain meteorological data are of importance for the astronomical observations; atmospheric refraction depends upon the barometric height and the temperature; atmospheric transparency, an important factor in photometric observations, is correlated with horizontal visibility; the measured variation of latitude depends to some extent upon the direction of the wind; the amounts of sunshine, of rainfall, and of clear sky at night give some indication of the general observing conditions. The astronomer has to make his observations at the bottom of a dense atmosphere, and it is only to be expected that atmospheric conditions can influence the observations in many different ways. The effects are often unsuspected and obscure in origin and may not be discovered until results are analyzed; it was, for instance, quite unsuspected in advance that the measured latitude would depend upon the direction of the wind, though not upon its velocity. With a complete record of meteorological data, the basic data are available for any purposes of subsequent analysis. The Observatory makes continuous records of wind direction and pressure, of the total flow of air, of dry- and wet-bulb temperatures, of barometric height, of rainfall, of sunshine by day and of clear sky at night—the last being recorded by the trails of Polaris and of  $\delta$ -Ursae Majoris obtained with a small fixed camera pointing to the pole. Daily eye observations are made of the barometer, dry-bulb and wet-bulb thermometers, radiation and earth thermometers, of the amount of cloud and of visibility. Some of the instruments used, such as the anemometers, are not of the most modern type, but the long series of observations made according to a uniform plan and with the same instruments is of special value for climatology. The Greenwich series of observations does, in fact, hold a unique place in British climatology. The data are of importance for various statistical purposes, such as questions of public health, the occurrence of epidemics, etc.; the meteorological results are therefore sent weekly to the Registrar-General. Observations are communicated daily to the Meteorological Office.

Magnetic observations were commenced in 1840 at the same time as the meteorological observations. Though not closely related, the magnetic and meteorological observations have always been in the charge of one department of the Observatory. This was primarily a matter of administrative convenience, to keep the non-astronomical work separate from the astronomical.

When the magnetic observations were started, they were made visually every 2 hr. throughout the day and night, but on one day each month they were made at 5 min. intervals throughout the 24 hr. This severe and trying labour was eliminated by the introduction in 1848 of continuous photographic registration, which has been maintained ever since, though with various changes and improvements in the recording instruments. A century of photographic registration has therefore been completed; the records are stored at the Observatory and are of great value in a variety of investigations.



In 1923 it became necessary to remove the magnetic observations from Greenwich because of the plans for the electrification of the local railway system. A site was selected near Abinger, on the slopes of Leith Hill, in Surrey, where a new magnetic observatory was built and observations were commenced in 1924. At that time the absolute observations of horizontal intensity were made with the Kew magnetometer, and those of dip with the dip inductor, which had superseded the dip circles in 1913. A few years later coil magnetometers were introduced as the standard instruments for the absolute measurement of horizontal and vertical intensity. The Schuster-Smith coil magnetometer for the measurement of horizontal intensity was installed in 1927, and the Dye coil magnetometer for the measurement of vertical intensity in 1928. Both these instruments were constructed at the National Physical Laboratory and are on loan to the Observatory from the Laboratory. The potentiometers used in conjunction with them are checked from time to time at the National Physical Laboratory.

The Schuster-Smith coil magnetometer has proved greatly superior to the Kew unifilar magnetometer in both speed and accuracy. The speed of observation is particularly valuable when conditions are at all disturbed. The base-line values of the horizontal intensity magnetograph deduced from the absolute observations have an uncertainty of not more than  $1\gamma$ . The scatter of the base-line values of the vertical intensity magnetograph, deduced from the absolute observations with the Dye coil magnetometer is a little greater, but the uncertainty is only about  $2\gamma$  or  $3\gamma$ . This instrument is adopted as the standard for vertical intensity, the dip being deduced from the observed values of the vertical and horizontal intensities.

Absolute observations of declination are made several times every weekday, using for reference an azimuth mark whose azimuth is controlled by observations of Polaris; those of horizontal and vertical intensity are made daily, except Sundays. Frequent observations of horizontal intensity are made with the Kew magnetometer and of dip with the dip inductor; these observations serve as a general check on the observations with the coil magnetometers and are not otherwise used.

The Royal Observatory was the pioneer in using electrical coil instruments as standards; it is of interest to remark that a small systematic difference between the dip inferred from these observations, and the dip measured directly with the dip inductor was traced to an unsuspected defect in the inductor, arising from slight play in the bearings of the rotating coil.

The recording variometers, which record declination, horizontal intensity, and vertical intensity, are of the well-known la Cour type. They include both slow-run and quick-run variometers. Records are also obtained with declination and horizontal intensity magnetographs of low sensitivity, which are of value in following the field changes during great magnetic storms when the large rapid movements cannot always be followed with certainty on the normal records. The published data include the hourly means of each element throughout the year, together with the monthly mean hourly values and the means for the five international quiet days and the five international disturbed days each month; the daily mean and daily extreme values for each element, with the corresponding monthly means for all days, for the quiet days, and for the disturbed days; the mean diurnal in-

equalities for each month, for the year, and for winter, equinox, and summer, of declination, dip, horizontal intensity and for north, west, and vertical components, for all days, for international quiet days, and for international disturbed days separately; the harmonic components of the diurnal inequalities of north, west, and vertical components, for each month, for the year, and for the three seasons, for all days, quiet days, and disturbed days; together with mean monthly and annual values for all elements.

The daily magnetic character figures, and the 3-hourly range indices are assigned on the basis of the daily records and are communicated regularly to the international centre at De Bilt. As opportunity offers, the estimation of the 3-hourly range indices is being carried backwards through the long series of Greenwich records, providing data of fundamental importance in many geophysical investigations.

The Royal Observatory has for some 30 years assumed the responsibility for the preparation of the world magnetic charts which are published by the Hydrographic Department of the Admiralty. Charts of declination are prepared at 5-yearly intervals; of horizontal intensity and of dip at intervals of 20 years. During the war, in connexion with the degaussing of ships as a protection against magnetic mines, a world chart of vertical intensity was prepared. It has been decided that charts of horizontal intensity, of dip, of vertical intensity, and of total intensity will be prepared in future at intervals of 10 years, in accordance with a recommendation of the Association of Terrestrial Magnetism of the International Union of Geodesy and Geophysics. The preparation of these charts involves the collection and examination of magnetic observations and surveys made in all parts of the world; from these observations the secular change and the rate of change of secular change have to be inferred in order to reduce the observations to a common epoch and to extrapolate to the epoch for which the charts are prepared.

Since the untimely loss of the non-magnetic ship, the *Carnegie*, put an end to the long series of ocean magnetic observations undertaken by the Department of Terrestrial Magnetism of the Carnegie Institution, Washington, the magnetic data over some of the ocean regions, and particularly over the southern Indian Ocean, have become increasingly uncertain. Reports received from vessels of the mercantile marine were sufficiently concordant to justify some empirical corrections to the charts. The matter was discussed with the Hydrographer of the Navy and, as a result of representations made to the Board of Admiralty, the construction of a non-magnetic ship was decided upon. The ship, known as the R.R.S. *Research*, was in an advanced stage of construction at the time of the outbreak of war, when work had to be suspended. The possibility of completing the ship, except for the auxiliary engines, and of putting her into commission as a sailing ship is under consideration, though no decision has yet been reached.

The harmonic analysis of the world magnetic charts and the comparison between the observed and computed field at various points on the earth's surface can give some indication of areas where the charts are seriously in error. The charts for 1922 and 1942 were analyzed in this way, and in each case it was found that the computed position of the north magnetic pole was not in agreement with the adopted position, which was determined by Amundsen in 1904 and was in close agreement

with the position assigned by Ross in 1831. The discordance appeared to be too great to be attributable entirely to errors in the charts, and it appeared probable that the magnetic pole had moved appreciably from its position in 1904, in a direction somewhat to the west of north. When in 1945 a series of polar flights by the Lancaster aircraft *Aries* was being planned by the Empire Air Navigation School, there seemed to be an opportunity for obtaining some evidence on this question. The Commandant of the Empire Air Navigation School agreed that such observations as were possible in conjunction with the other objects of the flight should be undertaken. One of the flights actually made passed over the Amundsen position of the magnetic pole and another passed near the computed position. The results of these flights provided strong supporting evidence for the movement of the magnetic pole. More recent observations made by the Canadian Eastern Arctic Patrol have fully confirmed the displacement, though not by so large an amount as the harmonic analysis had suggested.

Since the termination of magnetic observations at the Kew Observatory, the Royal Observatory has taken over the responsibility for the testing and certification of magnetic instruments of different types. This work is undertaken not only for Government Departments and Colonial Governments, but also for various institutions and commercial firms.

#### THE SOLAR DEPARTMENT

The magnetic observations, which are of some importance for navigation, led to a further development, which has no connexion with navigation. About the middle of the nineteenth century it was discovered independently by Sabine, Lamont and Wolf that magnetic phenomena have a period similar to the 11-year sunspot period, which had been announced shortly before by Schwabe. It was also found that magnetic storms often occurred when there was a large spot near the centre of the Sun's disk. The relationship suggested the need for supplementing the magnetic observations by solar observations. The solar department was accordingly established by Airy in 1873. A photo-heliograph, in which the primary image of the Sun is magnified by an enlarging lens to give an image 8 in. in diameter, was installed, and the regular daily photography of the Sun, whenever conditions permitted, was commenced. These observations have been continued without interruption. Photographs with a similar instrument are made at the Cape Observatory and are forwarded to Greenwich. There are normally but a few days in the year which are not represented in the combined Greenwich and Cape series; photographs for the missing days can usually be obtained on request from either the Kodaikanal Observatory or the Mount Wilson Observatory. The positions and areas of sunspots and faculae appearing on each photograph are measured. A general catalogue of sunspots and ledgers both of recurrent and of non-recurrent groups are prepared from the measures. The total areas of umbrae, of whole spots, and of faculae for each day are computed, as well as the mean areas and heliographic latitudes for spots north of the equator, for spots south of the equator, and for all spots. The collected results provide the most complete information that is available about

sunspots and faculae, and one of importance in the study of the relationships between solar and terrestrial phenomena.

The sunspot data have been used at Greenwich in a variety of investigations. The position of the Sun's axis was determined from the observations of sunspots in the period 1874 to 1912, using both recurrent groups and groups observed for eight or more days; the data were analyzed for three complete spot cycles separately, for four different phases of the cycle, and for the three chief zones of heliographic latitude. The inclination of the Sun's axis of rotation to the ecliptic was found to be  $7^{\circ} 10' \cdot 5$ , and the longitude on the ecliptic of the ascending node to be  $73^{\circ} 46' \cdot 8$  (epoch 1850.0). The motions of recurrent spots observed in five complete spot cycles, from 1878 to 1933, have been analyzed to determine the Sun's rotation period and its dependence upon heliographic latitude. The rotation periods derived from the five separate cycles were in excellent agreement, in contrast to the large secular change given by spectroscopic observations; the sunspots, however, because of their cyclic fluctuation in frequency, cannot be used to determine the rotation period year by year.

Many investigations have been made at Greenwich of the relationships between sunspots and terrestrial magnetic disturbances. The general statistical relationship between the occurrence of magnetic storms and the sunspot state of the central region of the Sun at the times of occurrence of the storms has been fully established. The largest storms tend to be associated with the largest spots; on the other hand, though the largest spots have a strongly marked tendency to persist for several rotations, the largest storms show little or no tendency to recur after 27 days—the period of the solar rotation—whilst moderate storms show a marked recurrence tendency. When a spot appears to be the source of a magnetic disturbance, the spot is usually situated, at the moment when the storm begins, between 2 days east and 4 days west of the Sun's central meridian.

In 1929 solar observations were extended to include visual observations of the Sun's disk in  $H\alpha$  light, using a spectrohelioscope lent by the Mount Wilson Observatory. The initial purpose of these observations was to detect any special disturbances on the Sun that might be related to the occurrence of magnetic storms. Measurements are made, with the line-shifter, of the radial velocities of dark markings and, in particular, of those in the neighbourhood of sunspots. The intensities of bright  $H\alpha$  flocculi and of prominences relative to the adjacent background are determined with a simple form of wedge photometer fitted with a comparison lamp. Visual measures of the contour of the normal Fraunhofer line  $H\alpha$  at the centre of the disk are also made.

A special study has been made of the bright chromospheric eruptions or solar flares. The sunspot activity was on the wane when the spectrohelioscope was installed. By 1936 sunspot activity, having passed a minimum, was increasing rapidly. The number of flares showed a correspondingly rapid increase. By 1937, when the number of flares had still further increased, the association between the flares and sudden fadings of short-wave radio transmissions, more particularly in the case of the larger and brighter flares, had been fully established. The simultaneity of the two phenomena implied that the radio fadings were due to a solar agency

travelling with the speed of light. A direct comparison photometer to enable the peak intensities of the flares to be rapidly measured in relation to the adjacent continuous spectrum at  $15\text{\AA}$  from  $H\alpha$  was constructed in the workshop and installed in 1939.

The flares are found to occur mainly in the vicinity of large sunspots when in the stage of active development. In a number of instances the full sequence of phenomena has been observed; the brilliant eruption with the synchronous radio fade-out, accompanied by a typical bay or crochet on the magnetic trace, followed at an interval of the order of 1 day by a great magnetic storm. The solar influence on geomagnetic disturbance has led to the study of various geomagnetic phenomena. It is found that there is a marked diurnal variation in the times of sudden commencements, with a minimum at about 8 to 9 hr. G.M.T. A small proportion of sudden commencements have the initial movement in a direction opposite to the normal; these 'reversed' sudden commencements show an entirely different diurnal frequency, with a maximum at the time when the normal sudden commencements show minimum frequency.

The Brentwood radio station of Cable and Wireless Ltd. reports direct to the Royal Observatory any radio fade-out while it is in progress. Ionospheric data are sent regularly to Greenwich by the Superintendent, Radio Research Station, Slough, the Engineer-in-Chief, Radio Branch, G.P.O., and the Controller (Engineering), B.B.C. Information about sunspots and flares observed at Greenwich is supplied to various radio research centres, while an informal liaison over solar observations in general has been maintained with the Radio Group of the Cavendish Laboratory, Cambridge; the Radio Research Station, Slough; and the operational Research Group of the Ministry of Supply. It is of interest to note that though the solar observations were commenced at Greenwich purely because of the scientific interest in the possible relationship between solar phenomena and geomagnetic disturbance, the observations have become of practical value for the forecasting of ionospheric conditions. At the same time, they are of increasing importance for the theoretical investigation of the processes involved.

#### ASTROMETRY AND ASTROPHYSICS DEPARTMENT

In this department is included a wide range of investigations in astronomy which have developed from the application of photography to astronomy. It includes two main sections: (i) astrometry, involving precise measures of positions of star images on photographic plates, which may be purely differential, involving small displacements in position between two different epochs, or may be used to derive absolute positions, by using a number of stars as reference points whose positions have been separately determined by meridian observations; (ii) astrophysics, which is concerned with physical characteristics, brightness, colour, spectra, etc.

The first development of the work at Greenwich in this direction was the participation in the great international project, proposed in 1887, of a photographic chart and catalogue of the entire sky. A large number of observatories shared in this project, which included the preparation of a catalogue, giving the positions of

all stars down to a certain brightness, and the publication of star charts showing all the stars to a fainter limit of magnitude. To each observatory there was allotted a certain region of the sky, the Greenwich Observatory assuming responsibility for the cap of  $25^\circ$  radius around the north celestial pole. 13 in. photographic refractors of the same focal length, having a scale on the plates of 1 mm. to 1' of arc, each plate covering a field of  $2 \times 2^\circ$ , were to be used. The astrographic refractor for Greenwich was made by Grubb of Dublin, similar telescopes being made for a number of other observatories. For the determinations of position, all the stars in the Greenwich zones down to the limit of magnitude  $9^m.0$  were observed with the transit circle in the years 1897 to 1905, while the measurement of the rectangular co-ordinates of the star images on the photographic plates was in progress. During these measurements, the diameters of the star images on the photographic plates were estimated in order to derive photographic magnitudes of the stars by the use of an empirical relationship connecting diameter and magnitude. In addition, the photographic magnitudes of all the stars brighter than  $9^m.0$  were determined with a Cooke triplet lens camera of 6 in. aperture and 27 in. focus, covering a large field without appreciable distortion. Each region was photographed when at the altitude of the pole, the polar region being also photographed on the plate, so that the magnitudes of the field stars could be derived by comparison with the standard north polar sequence of magnitudes, which had been determined at the Harvard Observatory. The sequence of exposures was arranged so that the mean time of exposures on the field was in close agreement with the mean time of exposures on the pole, the assumption being made that the atmospheric absorption was equal under these circumstances for the two regions at the same altitude. This very large programme of work was spread over a number of years. The project was an ambitious one and proved to be beyond the power of some of the co-operating observatories, so much so that it has not even now been brought to completion after the lapse of more than half a century. The Greenwich section, both of the chart and of the catalogue, was one of the first to be completed. The results are published in rectangular co-ordinates, with sufficient data to enable the right ascension and declination of any star to be readily derived. For all the stars, however, down to magnitude 9.0 on the scale of the *Bonn Durchmusterung*, together with all fainter stars included in the catalogues of the Astronomische Gesellschaft and in Carrington's circumpolar catalogue, 16,780 stars in all, right ascensions, declinations, and photographic magnitudes were published in a separate volume.

Many programmes of observation undertaken at Greenwich since the completion of this work have been planned in order to make the available information about the stars in the polar cap, from dec.  $+64^\circ$  to the north pole, more complete. The proper motions of the stars for which more than one position had been determined were derived by the comparisons of all available catalogues, each catalogue being reduced to a common basis by the application of systematic corrections. Then, commencing in 1923, the whole region was rephotographed with the astrographic telescope for the determination of relative proper motions of the stars. This second series of plates were exposed through the glass, so that by placing the corresponding plates of the two series of photographs film to film, the two images of each star were

brought into close proximity. It was necessary only to measure the small displacements between corresponding images differentially, and to apply corrections for differences in scale and orientation of the two plates in order to derive the relative proper motions. Comparisons between the photographic proper motions and proper motions based on meridian observations, where the latter were available, provided the data for converting from relative to absolute proper motions. The probable error of the derived proper motions was about  $\pm 0''.8$  per century in each co-ordinate. The value of the *Greenwich Astrographic Catalogue* as a source of stellar positions was greatly enhanced by the determination of the proper motions.

The astrographic telescope has been used for a variety of other investigations. Mention may be made of a special determination of the magnitudes of the stars in the standard north polar sequence, using a coarse wire grating to give sensibly round first diffracted images, with a calculable difference in magnitude from the central image. The magnitudes of the sequence had been determined at the Harvard and Mount Wilson Observatories, but there was an appreciable difference in scale between the two determinations. The investigation at Greenwich proved that the Mount Wilson scale was correct. The telescope also co-operated, along with the 26 in. refractor, in the two international programmes of observation of Eros at the favourable oppositions of 1901 and 1931 for the determination of the solar parallax.

The main programme of observation with the 26 in. photographic refractor, which was presented to the Observatory by the distinguished surgeon, Sir Henry Thompson, has been the measurement of stellar parallaxes. Such observations demand great care and precision and, before the commencement of the work at Greenwich, had been made mostly in the United States with telescopes of much greater focal length. The high latitude of Greenwich is not favourable for stellar parallax work, because the short nights in the summer make it impossible to secure observations near the times when the parallax factors are at a maximum; more photographs are needed for the same weight in the parallax determinations than in lower latitudes. The weather at Greenwich, moreover, makes it difficult to obtain a proper balance in the observations at the several epochs at intervals of about six months which are required. By special care in the adjustment of the lenses of the objective, controlled by photographs at intervals to detect any tilt or eccentricity, and by other precautions, the results have proved to be of an accuracy comparable with that given by longer focus telescopes.

The observations have been confined to stars in the Greenwich astrographic zones. The observing lists include all stars in this region of magnitude 5.5 or brighter; stars down to 7<sup>m</sup> with annual proper motions greater than  $0''.10$ ; stars down to 8<sup>m</sup> with proper motions greater than  $0''.15$ ; fainter stars with proper motions greater than  $0''.20$ , together with stars of type K0 with proper motions greater than  $0''.05$ , the latter being included to obtain information about the distribution of these stars in absolute magnitude. The photographs are obtained with the use of one of a series of rotating sectors, the aperture being chosen to reduce the magnitude of the parallax star to about 11<sup>m</sup>.5, the magnitude of the stars selected as comparison stars. For the brightest stars sufficient reduction in magnitude cannot be obtained by using a rotating sector; local desensitizing of the central region of

the plate with copper sulphate was tried for a time, in combination with a rotating sector, but the magnitude reduction produced by the desensitizing was uncertain. A neutral filter, giving a magnitude reduction of about 5<sup>m</sup> was therefore employed, in combination with a suitable sector.

The observations were at first made by Kapteyn's method, in which the plate is exposed at one epoch, then stored undeveloped, and exposed again at the next epoch, the small displacements between the two series of images being measured. The method resulted in much loss of weight; good definition at one epoch might be followed by bad definition or exposures interrupted by cloud at the second. There were difficulties in balancing the different epochs. The method was therefore abandoned and each plate developed after exposure at the one epoch. For the measurement of the series of plates for the determination of the parallax of a star, suitable comparison stars were selected; a blank glass plate was then ruled with short fine parallel lines near the position of each comparison star, and of the parallax star. A plate was placed film down on the ruled plate, and the small displacements between each star and the rulings were measured. The ruled plate served as a dummy, which permitted of accurate setting of the micrometer wire and with which each stellar photograph was compared, thereby making possible the intercomparison between the stellar photographs themselves. Three separate exposures were normally given on each plate during the first series of observations. The practice was then introduced of giving two exposures, the plate being turned through 180° between the exposures; this procedure reduces any errors that may be caused by small local film distortions. It has been found that two exposures, with intermediate reversal, give the same accuracy as three exposures without reversal and with the advantage of saving time at the telescope. About 750 determinations of stellar parallax have been made since observations were commenced.

The 26 in. refractor has been used for a number of smaller programmes. Several series of photographs of Jupiter's satellites were obtained at the request of Professor de Sitter, to provide material for his determination of the elements of the orbits of the satellites and for his investigation into the theory of their motions. Photographic magnitudes of stars down to magnitude 14 in a number of Kapteyn's Selected Areas, zones +15°, +30°, +45°, and +60° declination, were determined by comparison with the north polar sequence; the magnitudes of 395 stars within 1° of the north pole were also measured.

A 30 in. reflector is mounted on the same mounting as the 26 in. refractor; the arrangement is not convenient, as it is never possible to use the two telescopes at the same time and there can be inconvenient competition between the demands on the same mounting for different programmes of work. The reflector has been used for the photography of comets and other celestial objects; in particular, a very fine series of photographs of Comet Morehouse 1908, whose tail changed markedly from night to night, was secured. On photographs taken with it, the eighth satellite of Jupiter was discovered by Melotte. The reflector was used also for a programme of determination of effective wave-lengths of stars in the north polar cap. Using a coarse wire diffraction grating over the end of the telescope, the distance between the two first diffracted images depends upon the grating interval, upon the focal



length and upon the wave-length of the light. The first diffracted images are really short spectra. The distance between the points of maximum photographic intensity determines the colour or effective wave-length of the star. The points of maximum intensity depend upon the distribution of light in the spectrum of the star, and upon the intensity curve of the photographic plate. The use of a reflector avoids difficulties from chromatic aberration. The growth of the diffracted images is not the same for stars of different colours, so there is an exposure-time effect, which must be allowed for. The image given by a 10th magnitude star with an exposure of 10 min. was used as standard. The change of effective wave-length with spectral type of the star depends upon the type of plate employed; with panchromatic plates and a yellow screen the change was found to be approximately linear, whereas with blue-sensitive plates there was little change between A0 and F8. The dispersion in the first diffracted images in this investigation was comparable in amount with atmospheric dispersion at a zenith distance of about  $60^\circ$ ; the results obtained throw much light on the relative displacements for stars of different types due to atmospheric dispersion, which may introduce systematic errors into the determination of the solar parallax from observations of the minor planet Eros or of any other asteroid.

In 1931, Mr William Johnston Yapp offered to present a large telescope to the Observatory. A 36 in. reflector was decided upon. The telescope was brought into use in 1934 for the continuation of a programme of observations of the colour temperatures of stars which had been commenced with the 30 in. reflector. The programme was a particularly difficult one to undertake at Greenwich, where the atmospheric transparency is poor, variable, and not uniform in amount in different directions. The colour temperature of a star is the temperature of a black body which has the same relative distribution of energy throughout the spectrum as the star. The determination of colour temperature involves comparison with a terrestrial source whose colour temperature is known; it is convenient, however, to divide the investigation into two parts, intercomparing the stars and then making comparisons with the terrestrial source. A selection was made of twenty-five stars, of spectral types A and B and fairly evenly distributed, to serve as standard stars. These standards were intercompared one with another, when at the same altitude. Other stars were then compared with one or more of the standard stars.

The reflector was employed with a slitless spectrograph. At first a coarse wire diffraction grating with dispersion at right angles to that of the spectrograph was used to provide a photometric scale. But this was wasteful of observing time. A scale spectrograph was therefore used with multiple slits, whose breadths were closely in the ratio of 1:2:4, the dispersion being approximately the same as that of the slitless spectrograph. The exact ratios of the light passing through the three slits were determined by a half aperture method. A Lummer-Brodhun cube microphotometer was used for measuring the spectral intensities, measurements being made at eight points in the blue and at eight in the red, free from absorption lines.

As standard source of comparison, a standard acetylene burner, with a nominal colour temperature of  $2360^\circ$  K, was used. The burner was specially calibrated at the National Physical Laboratory. The difference of colour from the stars was reduced by the insertion of a blue filter in the beam from the burner, whose absorption was

measured with the scale spectrograph. The burner was placed at a distance of about 600 ft. on the Octagon Room roof. The horizontal reddening of the acetylene flame in this distance was determined by special observations.

The colour temperatures of most northern stars brighter than  $4^m.5$  and of spectral type A or earlier, as well as of many fainter stars of these types and of a selection of bright F- and G-type stars have been determined.

A slit spectrograph for use with the 36 in. reflector was completed in 1937. The optical parts are made of ultra-violet glass, and one-prism or three-prism dispersion can be used. The spectrograph was mounted towards the end of 1939, on the completion of the colour-temperature programme and various tests were made. But circumstances at that time made it impossible to commence any definite programme of observation. Since the war it has been employed in attempts to detect faint blue companions in late-type spectroscopic binary systems.

#### MISCELLANEOUS PROGRAMMES

Some items of the work of the Observatory do not come definitely within the scope of any particular department, but depend to some extent upon the personal interest of individual members of the staff. Expeditions have been sent from time to time to various parts of the world to make observations of total eclipses of the Sun, the programmes being determined by the problems of current importance. It was the expedition from Greenwich to Brazil for the observation of the total eclipse of 29 May 1919 which provided the first evidence to confirm Einstein's predicted displacement of stars in the vicinity of the Sun. The most recent expedition was a small expedition to Mombasa for the eclipse of 1 November 1948, to test a method of accurate determination of the position of the Moon, designed to be used on the occasions of a total eclipse at two widely separated centres, for the purpose of providing an accurate geodetic connexion.

The 28 in. refractor, installed in 1886, has been employed for many years for the observation of close double stars with a filar micrometer. The measures obtained up to 1919 were collected and published in a single volume. The observations were used, in conjunction with observations made elsewhere, for the determination of the dynamical parallaxes of 576 double stars. The parallax of a binary system of known period and angular dimensions of orbit can be calculated if the combined mass is known; in the absence of a knowledge of the mass, the error introduced by assuming the combined mass to be twice that of the Sun is relatively small, as the mass enters only to the power of  $\frac{1}{3}$ . The parallax so deduced is called the dynamical parallax. When the binary star has not been observed for a complete period, the parallax can be estimated, though with less certainty, from the rate of change of angle and distance.

A marked improvement in the accuracy of the measures followed from the introduction of a comparison image micrometer, constructed in the Observatory workshop. A Wollaston prism is used to give a double image of an artificial star, the separation of the two images being varied by altering the distance of the source from the prism. Rotation of the prism rotates the position angle of the artificial

PROSPECTUS SEPTENTRIONALIS.

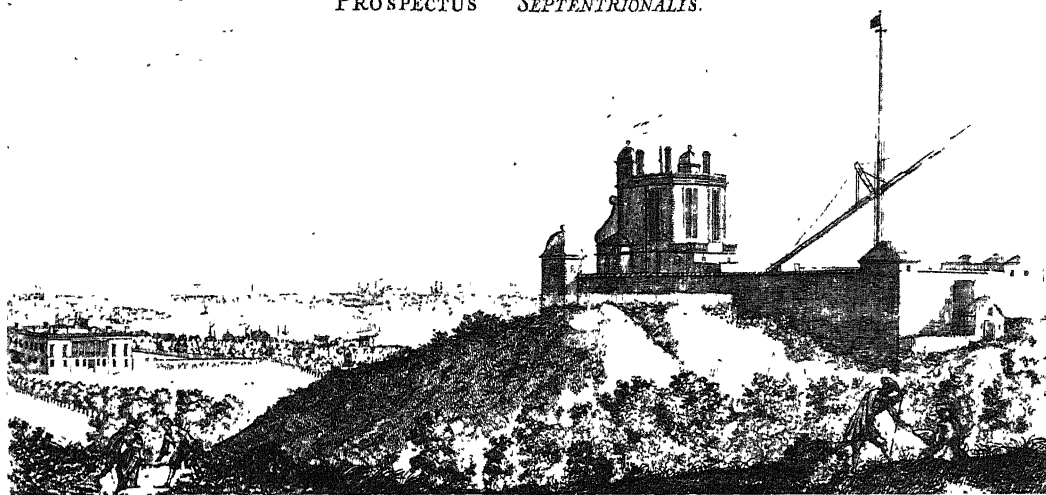


FIGURE 1. View of Flamsteed's original observatory (Wren building) looking north.



FIGURE 2. View from the 26-inch dome, looking towards the Wren building (circa 1930).

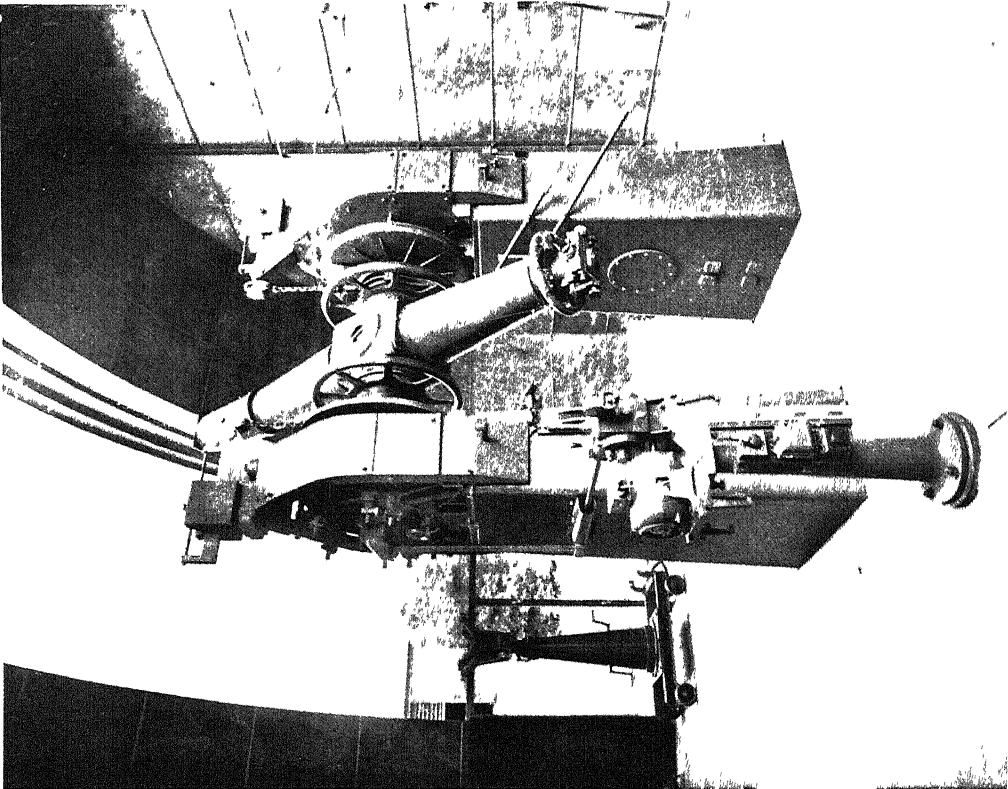


FIGURE 4. Reversible transit circle

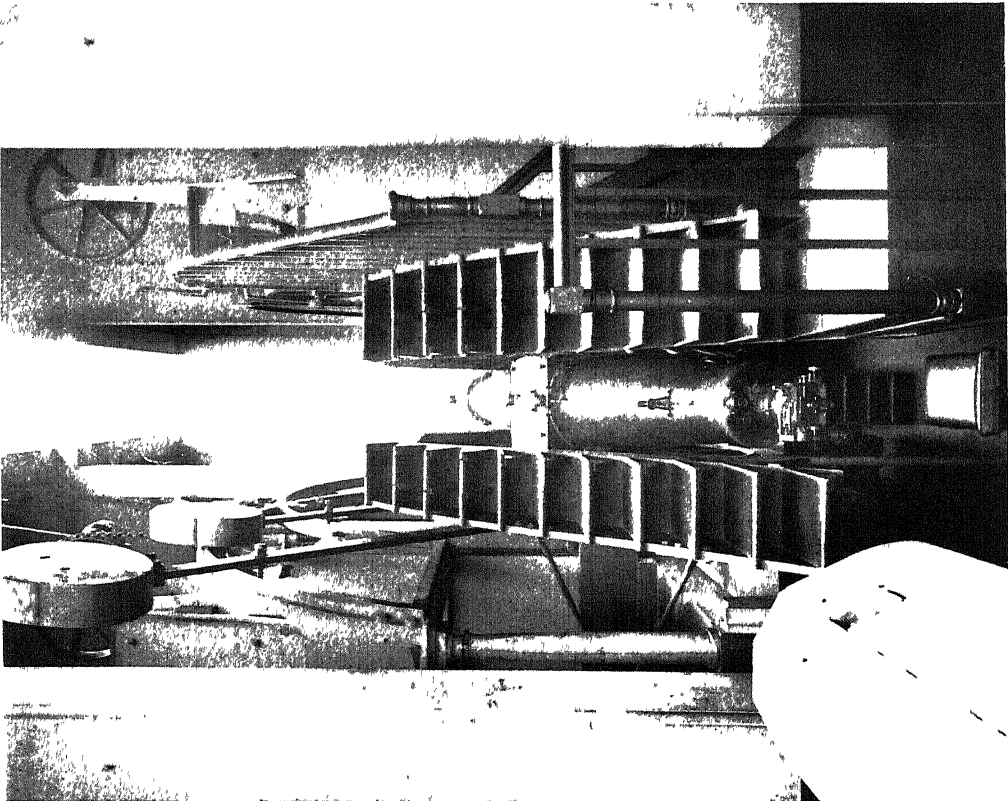


FIGURE 3. Any transit circle

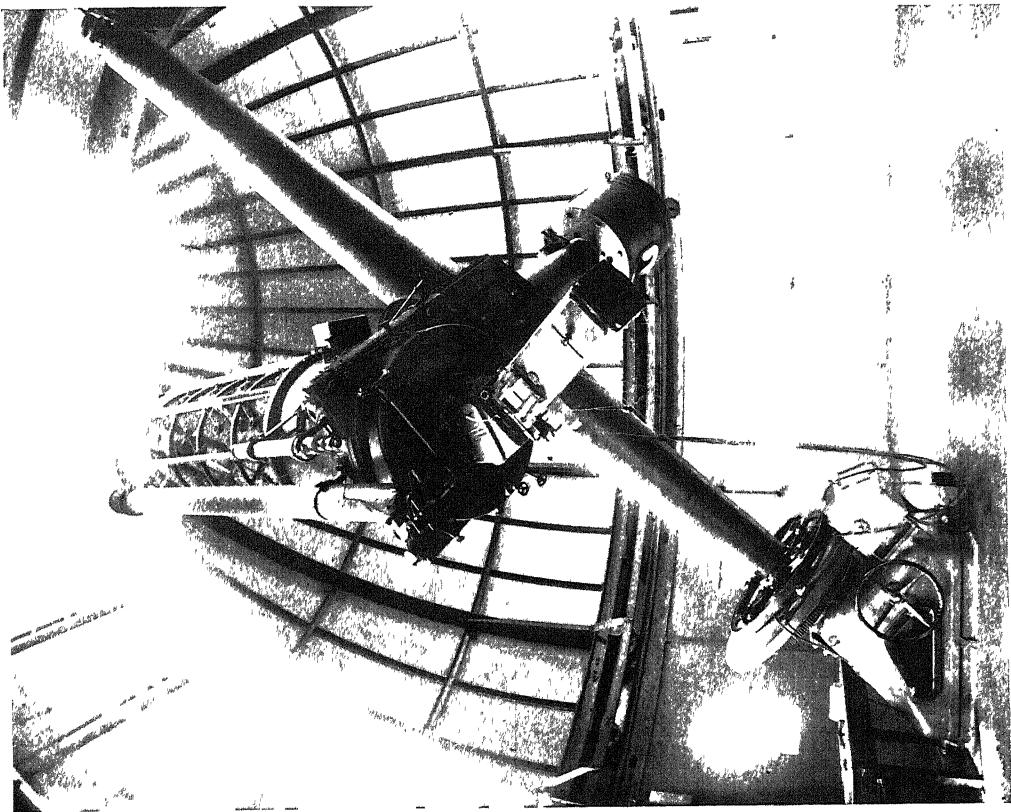


FIGURE 6. 30-inch reflector.

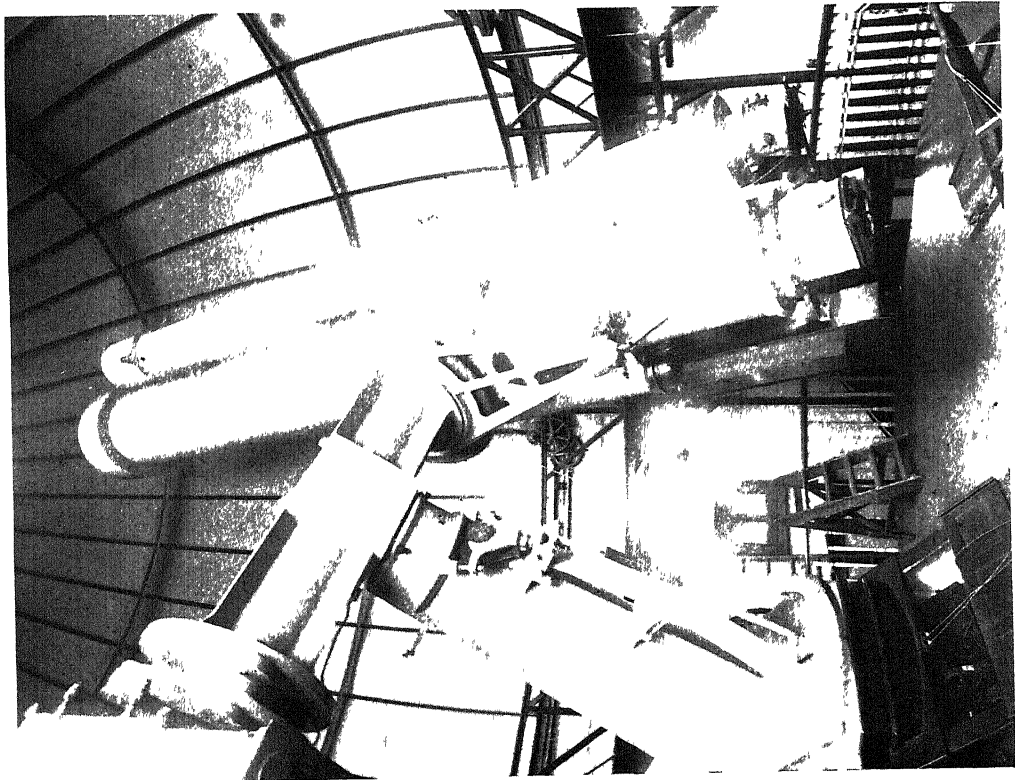


FIGURE 5. Thompson 26-inch reflector.

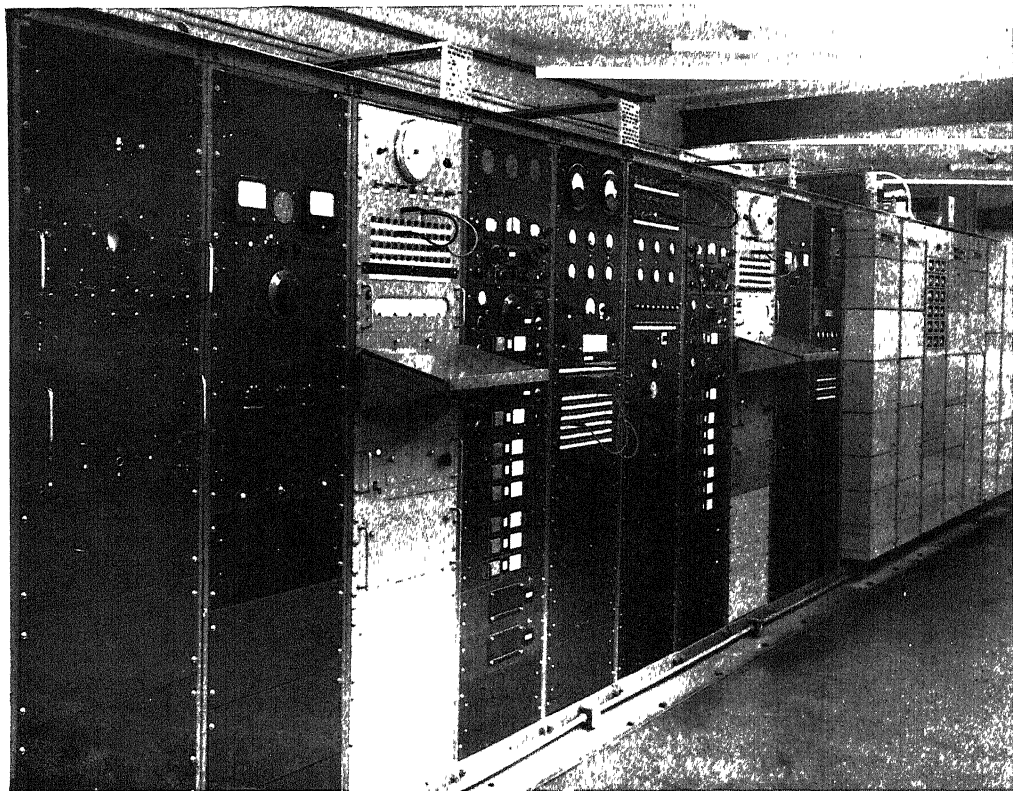


FIGURE 7. Control-room of Time Department.



FIGURE 8. General view of Herstmonceux Castle.



binary. Crossed nicols permit the brightness of either image to be varied. A blue filter is used to give a colour temperature of about  $5500^{\circ}$  K, so that the artificial images appear of about the same colour as the stellar images. The position angle, separation and magnitudes of the two images are adjusted to be comparable to those of the binary star; the four images, two real and two artificial, are set to form the corners of a parallelogram. The eye is sensitive to any slight lack of symmetry, even under unsteady conditions when the use of a filar micrometer is difficult. The comparison image micrometer is particularly advantageous in the measurement of close pairs and, because no field or wire illumination is required, it enables fainter stars to be observed.

#### CHRONOMETER DEPARTMENT

In 1821, soon after the control of the Royal Observatory passed from the Master-General of the Ordnance to the Board of Admiralty, the charge of chronometers used in H.M. Navy was transferred to the Royal Observatory. In the following year public trials of chronometers were instituted. Makers were invited to submit chronometers for trial, with a view to purchase by the Admiralty, and money prizes of considerable value were offered for the best chronometers. This system of annual trials was continued up to the beginning of the World War of 1914–18, though the giving of special prizes was discontinued after a few years. These trials had a considerable influence in stimulating improvements in the design and construction of chronometers.

Because increasing difficulty was found in getting chronometers and navigational watches satisfactorily repaired and adjusted by the trade, a Repair Shop was started in connexion with the Chronometer Department in 1937, where repairers and adjusters could be trained to the high degree of skill essential for work on precision time-pieces. The Repair Shop proved invaluable during the war, when the numbers of repairs to be dealt with was very large and when the Chronometer Department was testing and issuing an average of from 25,000 to 30,000 chronometers and watches a year. It is expected that the amount of repair work undertaken will gradually expand, and a scheme has recently been introduced for the indenturing and training of apprentices. The facilities of the Repair Shop are available also for any fine precision work required in connexion with the construction and modification of instrumental equipment.

#### OPTICAL AND ELECTRONIC LABORATORIES

The progressive expansion in the scope of the work of the Observatory has involved the employment of much specialized equipment, which has had to be designed in the Observatory and made in the Workshop. The slitless spectrograph, the scale spectrograph, and the Lummer-Brodhun cube microphotometer, employed in the colour-temperature programme, and the comparison image micrometer for double-star observations are a few of the many items of equipment which have been made at the Observatory. The satisfactory design of new equipment not infrequently requires a considerable amount of preliminary investigations and tests, and the

need for a definite laboratory section has progressively increased. The introduction of quartz crystal vibrators as standard clocks required the use of much ancillary electronic equipment; because much of the special equipment needed was not available commercially, it became necessary to design and construct it in the Observatory. A laboratory section has accordingly been formed, comprising both an optical and an electronics laboratory. Started initially for the special needs of the Time Department at the time when the work of that department was expanding rapidly, the laboratories can meet the requirements of all departments of the Observatory. Amongst the electronic equipment which has been designed and constructed, mention may be made of a special radio receiver for the reception of radio time signals transmitted on the long-wave band with low carrier frequency, designed to secure constant lag, a high degree of selectivity, and a steep build-up curve, and with provision for selecting any particular point on the build-up curve and for securing constant output voltage; of a 2 Mc./sec. standard frequency transmitter, controlled by one of the 100 kc./sec. oscillators; of a receiver for comparing the frequency of the Droitwich 200 kc./sec. transmissions with any of the Abinger standards; of a number of double current electronic send relays; and of a great deal of switching, modulating, and monitoring equipment.

#### NAUTICAL ALMANAC OFFICE

In 1766, the year after his appointment as Astronomer Royal, Maskelyne published the first number of the *Nautical Almanac* (for the year 1767). The *Almanac* was designed for the use of seamen and particularly to facilitate the employment of the method of lunar distances for determining longitude. It proved to be a most valuable aid to navigation. Maskelyne continued to produce it annually for 44 years, until his death in 1811. The computations for the *Almanac* were performed in duplicate by computers, mostly working at home, and it acquired a very well-deserved reputation for high accuracy. In 1781 Maskelyne published a volume of *Tables Requisite to be Used with the Nautical Ephemeris*, which was in effect a handbook for use with the *Almanac*. Pond, who succeeded Maskelyne as Astronomer Royal, did not take the same interest in the *Nautical Almanac*, though he remained nominally responsible for it. The *Almanac* lost its reputation for accuracy, and eventually in 1831 a separate Nautical Almanac Office was established with its own Superintendent and having no formal connexion with the Observatory.

In 1937, the Nautical Almanac Office was again placed under the direction of the Astronomer Royal and became a branch of the Royal Observatory, though retaining its own identity and its Superintendent. The *Almanac* at that time was responsible for the production and publication of the standard edition of the *Nautical Almanac* and of the *Abridged Nautical Almanac*, designed for navigational purposes. Shortly afterwards the office undertook the production of an *Air Almanac*, adapted to the special requirements of air navigation. The airman does not need to know his position as accurately as the sailor, but, because of the high speed of modern aircraft, he requires to deduce his position with the minimum of delay after making the observations. To meet these requirements, the data in the *Air Almanac* are



presented in a special way, Greenwich hour angle being used instead of right ascension, and to a lower degree of accuracy than in the *Abridged Nautical Almanac*. Special *Air Navigation Tables* were also prepared in the office and published, for use with the *Air Almanac*, to facilitate the rapid derivation of the position of the aircraft.

In 1940 the publication (for the year 1941) of an annual volume of *Apparent Places of Fundamental Stars* was commenced. This volume gives the apparent places, at 10-day intervals, for most stars, but at daily intervals for close circumpolar stars, of the 1535 stars in the FK3 Fundamental Catalogue; the time determinations at all observatories are based upon these positions. The computations of the apparent places are shared by the United States, France, Germany, and Spain; the Nautical Almanac Office is responsible for the co-ordination of the work, for the collation of the data, and for the preparation and publication of the volume.

At the Conference of Commonwealth Surveys in London in August 1947, a strong desire was expressed for a special almanac to be prepared and published to meet the needs of topographical surveyors. Detailed proposals were therefore prepared in the office for a *Star Almanac for Land Surveyors* and have been approved. The first issue will be made in 1950 for the year 1951.

The experience gained with the *Air Almanac* has confirmed the advantages of the method of tabulation of data according to Greenwich hour angle, and has given rise to a desire for the revision of the *Abridged Nautical Almanac*. Various alternative arrangements of presentation of the data have been considered, and, after much detailed consultation with all classes of users, the final form has been settled. In its revised form, which will be issued in 1951 for the year 1952, the *Almanac* will tabulate Greenwich hour angle in arc directly, instead of right ascension.

Special investigations into methods and tables for both sea and air navigation have been made, including a comprehensive survey of tables for astronomical polar navigation. The office has also been responsible for the preparation and publication of various tables, including *Seven-Figure Trigonometrical Tables for Every Second of Time* (1939), *Five-Figure Tables of Natural Trigonometrical Functions* (1941), *Planetary Co-ordinates for the Years 1800–1940, Referred to the Equinox of 1950.0* (1933), and of a continuation volume for the years 1940–60 (1939).

The office has been a pioneer in the adaptation of computations, formerly performed by logarithms, to machines. Because of its wide experience in methods of numerical computation and its machine equipment, it was able to provide a computing service to deal with a great variety of problems for various Government departments, which presented themselves during the war. Much preliminary investigation was often needed to discover the best method of solving special problems, with the least expenditure of labour and of time. Approval has recently been given for the installation of a complete range of Hollerith punched-card equipment, suitable for general computational work. It is intended to extend the use of the equipment, where suitable, to the work of the Observatory as a whole, as well as to the more routine work of the office. It is also hoped, eventually, to produce copy for some of the office publications automatically on card-operated machines.

The office has a close link with navigational problems and maintains a complete library of the navigational almanacs and tables of all countries. It is at present engaged on the computational work necessary for the latticing of charts required for the Decca system of navigation.

#### REMOVAL OF THE OBSERVATORY FROM GREENWICH

The conditions at Greenwich for astronomical observations have progressively deteriorated as London has grown outwards beyond the Observatory. The increasing pollution of the atmosphere and the increasing brightness of the sky at night have combined to affect adversely the quality and nature of the observations. Photometric and spectrophotometric observations, which require a uniform transparency in different directions and freedom from rapid variations of transparency, are practically impossible when clouds of smoke from nearby power stations and factories drift over the Observatory. But every type of observation is affected—meridian, solar, visual and photographic; in the exacting work of double star observations it has become impossible to observe close doubles which were observed with relative ease half a century ago. Under such conditions, the removal of the Observatory from Greenwich was essential if the Observatory was to continue to make useful contributions to astronomy. With the strong support of the Board of Visitors, proposals to remove the Observatory to a new site were submitted to the Board of Admiralty shortly before the outbreak of war. The war started before a decision was reached, and the question of removal had then to be deferred. During the war the principal instruments were partially dismantled, the Time Department was transferred to Abinger, and the Chronometer Department, with the Repair Shop, was moved first to Bristol and then to Bradford-on-Avon. The work of the Magnetic Observatory was becoming hampered by disturbances from the extension of railway electrification, and proposals for its removal to a site remote from railways were made. Widespread search for suitable new sites was carried out. After the termination of the war, the question of removal was again taken up. A short list of the most promising sites was prepared, and these sites were visited by a Committee of the Board of Visitors. Finally, it was announced on 11 April 1946 that Herstmonceux Castle in Sussex had been chosen as the future home of the Royal Observatory, and approval was given for the transfer of the Magnetic Observatory to a site to be selected in north Devon at a distance of at least 10 miles from any railway.

Some 372 acres of ground were acquired with the fifteenth-century castle, providing adequate space for erecting the various telescopes and for future additions to the equipment, and a safeguard against near encroachment by undesirable developments. A first stage of the removal is in progress. The Chronometer Department and the Secretariat have moved to Herstmonceux. A solar building to house the photoheliograph, two spectrohelioscopes, and spectrographic equipment for solar research is nearing completion. The transfer of the Solar Department, of the Magnetic and Meteorological Department, and of the Nautical Almanac Office should be possible during the course of the present year. Further stages of the removal, involving the erection of buildings and domes for telescopes, are under

consideration. The 26 and 28 in. refractors, whose domes were seriously damaged during the war, have been dismantled; the opportunity is being taken for some alterations to be made to these telescopes before re-erection takes place. The work of some departments of the Observatory is necessarily on a reduced scale during the present period of transition. There are, however, great hopes for the future, when the Observatory has been fully established in its new home and can reap the advantage of the good observing conditions. A selection of several possible sites in north Devon for the Magnetic Observatory has been made, and tests of their freedom from magnetic anomalies have been made. Some further sites will be examined before a definite selection is made.

On the occasion of the commemoration of the tercentenary of the birth of Sir Isaac Newton, held in London in July 1946, the President of the Royal Society announced that the Chancellor of the Exchequer had agreed to provide funds for the construction of a reflecting telescope of 100 in. aperture, to be associated with the name of Sir Isaac Newton and to be available for use by qualified astronomers from all observatories in Great Britain. It has been decided that the telescope will be erected in the grounds of the Royal Observatory at Herstmonceux. The telescope will be under the administrative control of the Astronomer Royal; a special Board of Management will be responsible for the scientific direction, including the designing of the telescope, the supervision of its construction, the consideration of programmes of observation, and the allocation of observing time between the various users of the telescope. The Board of Management will consist of the Astronomer Royal (Chairman), the Astronomer Royal for Scotland, and the Directors of the Cambridge and Oxford University Observatories as *ex-officio* members, together with four Fellows of the Royal Society and four Fellows of the Royal Astronomical Society. The telescope will enable British astronomers to undertake many programmes of observations which have hitherto been impossible because of the restricted light-gathering power of the largest telescopes at present in use in Great Britain, while the library, workshop, and other facilities of the Royal Observatory will be available to all users.

# On chlorocruorin and haemoglobin

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Chlorocruorin is a dichroic red-green respiratory protein. It is chemically similar to haemoglobin, and is only found dissolved in the blood of certain marine annelid worms.

Chlorocruorin is the characteristic blood pigment of the Serpulimorpha (serpulids and sabellids), but in the genus *Serpula* both chlorocruorin and haemoglobin are present together in the blood. This is the first time that two respiratory pigments have been found in the blood of one animal. Young individuals have relatively more haemoglobin, older ones more chlorocruorin.

Within the serpulid genus *Spirorbis*, one species has chlorocruorin in its blood, another has haemoglobin, while a third has neither pigment. As their habitats are similar, no functional explanation for these differences suggests itself.

The oxygen affinity of all chlorocruorins tested is considerably lower than that of most haemoglobins. But in *Serpula* the oxygen affinities of the chlorocruorin and haemoglobin are the same as one another.

The carbon monoxide affinity of chlorocruorin (in *Branchiomma*) is higher than that of any haemoglobin.

Although Serpulimorpha have chlorocruorin in their blood, the haem present in their tissues (muscles, eggs, sperm) is protohaem, not chlorocruoro-haem. One genus, *Potamilla*, with chlorocruorin in its blood, has haemoglobin in the muscles.

Chlorocruorin is known only from blood, and from the mucous tube of *Myxicola*; none has been found in cells. Coelomic fluid contains none.

Protohaem is secreted into the protective tubes of both serpulids and sabellids. A proto-haemochromogen is present in the gut fluid of serpulids, recalling that found in crustaceans and molluscs.

## 1. COLOURS AND SPECTRA

Four respiratory protein pigments are known, which undergo reversible oxygenation. These are haemoglobin, chlorocruorin, haemerythrin and haemocyanin. Haemoglobin is widespread in animals, chlorocruorin is restricted to a few marine annelid worms, haemerythrin has been found only in sipunculids, an annelid and a brachiopod (Kawaguti 1941), while haemocyanin occurs in decapod crustaceans, some molluscs and arachnids. All four pigments are found dissolved in body fluids, but only haemoglobin and haemerythrin occur in cells.

Chlorocruorin is dichroic, being red in concentrated, green in dilute solution. It is found only in the blood plasma of certain Polychaeta; these are Serpulimorpha (serpulids and sabellids), Chlorhaemidae and probably some Ampharetidae.\* Chemically, chlorocruorin is very nearly allied to haemoglobin (Fox 1926). Like the latter, it binds oxygen in a labile manner, with a ratio of two atoms of oxygen to one of iron (Fox 1934). Both haemoglobin and chlorocruorin contain a haem group, that is, a porphyrin united to an iron atom.† The two porphyrins differ only

\* Here the blood is of a dichroic red-green colour but the spectrum has not yet been studied.

† In spite of their names, neither haemerythrin (containing iron) nor haemocyanin (containing copper) has a haem group.

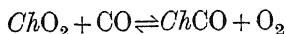
in one side-chain of one of their four pyrrole groups; the protoporphyrin of haemoglobin has two vinyl groups as side-chains, while in chlorocruoroporphyrin one of these two vinyls is oxidized to an aldehyde group (Fischer & Seemann 1936; Fischer & Deilmann 1944). Protohaem and chlorocruorohaem\* differ from one another correspondingly. Chlorocruorin differs from haemoglobin not only in the haem part but also in the protein part of the molecule (Warburg 1932).

There is no considerable colour difference between oxygenated and deoxygenated chlorocruorin as there is with haemoglobin. In strong solution oxychlorocruorin is of a purer red tint, chlorocruorin a browner red; in dilute solution there is less difference between the two, but the deoxygenated pigment is of a rather yellower green. The absorption spectra of oxychlorocruorin and chlorocruorin have already been described (Fox 1926), but it is necessary here to amplify the description. Deoxygenated chlorocruorin, when viewed with a spectroscope of small dispersion, shows a double band, instead of a single one as seen in haemoglobin. Measured with a Zeiss microspectroscope, calibrated by the  $585\text{ m}\mu$  line of neon, the axes of the  $\alpha$ - and  $\beta$ -bands of the oxychlorocruorin of *Sabella spallanzanii* (Viviani)† are at  $604$  and  $558\text{ m}\mu$ . Deoxygenated by a minimal quantity of sodium hydrosulphite, the two bands become much weaker but do not fuse into a single band; one remains at  $604\text{ m}\mu$ , and the other moves to about  $570\text{ m}\mu$ . The former is rather darker than the latter, not as shown in figure 5 of my 1926 paper. The light region between the maxima in the deoxy band hardly exists in the spectrophotometric curve (Fox 1926, figure 3) and is partly an optical illusion. It is none the less of practical importance, for the following reason. In respiration experiments with translucent invertebrates containing haemoglobin, the moment when the pigment finally loses its oxygen can be determined with a spectroscope by the disappearance of duplicity in the absorption band; this cannot be done with chlorocruorin.

## 2. CARBON MONOXIDE AFFINITY

Haemoglobin in the blood of vertebrate animals has a much higher affinity for carbon monoxide than for oxygen, and the carbon monoxide affinity varies with the kind of animal (Krogh 1910). Nothing has been known, however, up to the present of the carbon monoxide affinity of chlorocruorin.

The relative affinity of chlorocruorin (*Ch*), or of haemoglobin, for the two gases in the reversible reaction



is given by the equilibrium constant  $K$  in the equation

$$K = [ChCO]/[ChO_2] \times pO_2/pCO, \quad (1)$$

in which square brackets denote concentrations and  $p$  is pressure. From this it is clear that when the pigment is 50% in the carboxy state and 50% in the oxy state,  $K$  becomes the ratio of oxygen to carbon monoxide.

\* Incorrectly called 'Spirographishamin' by Warburg, Negelein & Haas (1930).

† Formerly called *Spirographis spallanzanii* Viviani; see Ewer (1946).

I have found the value of  $K$  for the blood of *Branchiomma vesiculosum* (Montagu), containing chlorocruorin, and for the bloods of *Arenicola marina* (L.), *Tubifex* sp., the larva of *Chironomus dorsalis* Meigen, *Planorbis corneus* L., the rabbit, the horse and man, all of which contain haemoglobin. The blood of each kind of animal was diluted in M/20-phosphate buffer at pH 7.3 and then centrifuged and filtered to give a limpid solution. Some of this was put into a Thunberg tube fitted with a 2 cm. optical cell. The concentration was adjusted to give an optical density,  $\log_{10} I_0/I$ , of about 1.5 at the  $\alpha$ -band, measured with a Hilger-Nutting visual spectrophotometer. After equilibration at 20° C with air in the case of haemoglobin, or oxygen for chlorocruorin—which cannot be saturated with oxygen at atmospheric partial pressure (Fox 1926, 1932)—the optical densities were determined at wave-lengths 561 and 578  $m\mu$  for haemoglobin, or 590 and 606  $m\mu$  for chlorocruorin. Previous plotting of the absorption curves of the oxy and carboxy compounds had shown that the two wave-lengths mentioned for each pigment are those at which the light absorption differs most. The Thunberg tube was then evacuated, filled with a known mixture of oxygen and carbon monoxide in a ratio which would give about a half-and-half mixture of the two compounds of the pigment, and the solution equilibrated with the gas by rotation for half an hour in a water thermostat at 20° C in dim light. After this, the changed optical densities were determined at the same two wave-lengths. When this had been done, the tube was again evacuated, filled with pure carbon monoxide, and re-equilibrated, after which the two densities were again measured. From the six density measurements, the relative concentrations (square brackets below) of the oxy and carboxy compounds in the solution in equilibrium with the gas mixture can be calculated as follows. If  $d_1, d_2$  are the optical densities of the mixture of oxy- and carboxychlorocruorin,  $e_1, e_2$  those of oxychlorocruorin, and  $f_1, f_2$  those of carboxychlorocruorin, at wave-lengths  $\lambda_1, \lambda_2$  respectively, then

$$\frac{d_1}{d_2} = \frac{e_1[ChO_2] + f_1[ChCO]}{e_2[ChO_2] + f_2[ChCO]},$$

and therefore

$$\frac{[ChCO]}{[ChO_2]} = \frac{d_1 e_2 - d_2 e_1}{d_2 f_1 - d_1 f_2}.$$

From this the value of  $K$  in equation (1) is directly deducible.

The following are the values of  $K$  obtained: *Branchiomma* 570, *Chironomus* 400, horse 280, man 230, *Arenicola* 150, *Tubifex* 40, *Planorbis* 40, rabbit 40. From five to eleven determinations were made for each type of animal. The separate values of  $K$  obtained for each varied considerably, and as there were not enough values to give standard errors, the means have been rounded to the nearest 10. For the haemoglobin of human blood,  $K$  has previously been found to be 235 or 246 (Douglas, Haldane & Haldane 1912), 240 (Killick 1948) or 490 (Anson, Barcroft, Mirsky & Oinuma 1924). My value is sensibly the same as the first three of these. Anson *et al.* agree with me in placing the horse above man and the rabbit much lower. It is seen that  $K$  for the chlorocruorin of *Branchiomma* is higher than that for any haemoglobin. The haemoglobins differ much among themselves, and some of them are known to have values of  $K$  below the lowest given above. Values for the muscle haemoglobin of the horse are 14 to 51 (Theorell 1934; Roche 1933) and for the

cellular haemoglobin of the horse bot-fly larva (*Gastrophilus*) 0.67 (Keilin & Wang 1946).

The diverse values of  $K$  cannot be related to other properties of the respiratory pigments. *Chironomus* has the highest oxygen affinity of a blood haemoglobin (Fox 1945) with the highest value of  $K$  for any haemoglobin. The horse's muscle haemoglobin, on the other hand, has a higher oxygen affinity than that of the horse's blood, but a much lower  $K$ . Chlorocruorin, with the highest  $K$ , has a very low oxygen affinity (p. 383). There is no relation between  $K$  and the span, which is the difference between the wave-lengths of oxy and carboxy  $\alpha$ -bands, as was once thought (Keilin & Wang 1946).

### 3. BLOOD PIGMENTS IN THE SERPULIMORPHA

Chlorocruorin is the characteristic blood pigment of the Serpulimorpha.\* In another group of Polychaeta, the Ampharetidae, there are several genera with green and one (*Melinna*) with red blood. Among the Serpulidae there is one genus, *Spirorbis*, in which one species, *S. borealis* Daudin, has chlorocruorin, another species, *S. corrugatus* (Montagu), has haemoglobin as its blood pigment, while a third one, *S. militaris* (Claparède), has neither pigment; its blood is colourless. That chlorocruorin or haemoglobin is really absent in this blood, not just dilute, is shown by the benzidine reaction being negative in the blood vessels, while it is positive for the other two species. *S. corrugatus* and *S. militaris* are found on seaweeds and on stones respectively, at the same depth of 3 to 4 m. at Naples, and I can suggest no functional reason for the presence or absence of haemoglobin in them, nor for chlorocruorin instead of haemoglobin in *S. borealis* which is common on the seaweed *Fucus* on our coasts.

The genus *Serpula* proves to be unique in the animal kingdom in having two respiratory pigments in the blood; it possesses both chlorocruorin and haemoglobin. The blood is greenish brown in colour. It has three strong absorption bands in the visible region of the spectrum. These are the  $\alpha$ -band of oxychlorocruorin and the  $\alpha$ - and  $\beta$ -bands of oxyhaemoglobin. The relatively weak  $\beta$ -band of oxychlorocruorin is not seen; it merely slightly obscures the bright region of the spectrum between the oxyhaemoglobin  $\alpha$ - and  $\beta$ -bands. The wave-lengths of the axes of the bands in diluted shed blood are  $604\text{ m}\mu$  for  $\alpha$ -oxychlorocruorin,  $577\text{ m}\mu$  for  $\alpha$ -oxyhaemoglobin and c.  $541\text{ m}\mu$  for  $\beta$ -oxyhaemoglobin. When the blood is treated with pyridine and sodium hydrosulphite, the presence of both chlorocruorohaem and protohaem, derived from the two respiratory proteins, is demonstrated by the appearance of the two haemochromogens, with their  $\alpha$ -bands at 583 and  $557\text{ m}\mu$  respectively.

\* I have found chlorocruorin to be the blood pigment of the following Serpulimorpha. Serpulidae: *Hydroides norvegica* (Gunnerus), *H. uncinata* (Philippi), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Filograna implexa* Berkeley, *Salmacina incrustans* Claparède, *Protula tubularia* (Montagu), *P. intestinum* L., *Apomatus similis* Marion, *A. ampulliferus* Philippi, *Spirorbis borealis* Daudin. Myxicolidae: *Myxicola infundibulum* (Rénier), *M. aesthetica* (Claparède). Sabellidae: *Sabella pavonina* Savigny, *S. spallanzanii* (Viviani), *Potamilla reniformis* (O. F. Müller), *P. stichophthalmus* (Grube), *Branchiomma vesiculosum* (Montagu), *Dasychone bombyx* (Dalyell), *D. lucullana* (Della Chiaje), *Amphiglena mediterranea* (Leydig), *Dialychone acoustica* Claparède.

The duplicity of respiratory blood pigment is characteristic of the genus *Serpula*, for it occurs both in *S. vermicularis* L. and *S. lobiancoi* Rioja.\*

The ratio of haemoglobin to chlorocruorin varies with size, that is, with age, in the blood of individual *S. vermicularis*. There is more haemoglobin in the blood of young, more chlorocruorin in older animals. If chlorocruorin, so rare in the animal kingdom, were regarded as a mutation of the widespread haemoglobin, the higher proportion of the latter in young *Serpula* could be looked upon as a case of recapitulation. Table 1 gives the data. It is seen that the larger animals have relatively more chlorocruorin, but that with one and the same body length there may apparently be considerable differences in the ratio of the two pigments. No doubt this is due largely to individual variation. But it must also be due in part to two errors. The relative intensities of the  $\alpha$ -bands were judged with the Zeiss micro-spectroscope by eye: this judgement cannot be very precise. Indeed, in the case of the smallest worms the oxyhaemoglobin band was sometimes perhaps really 4 times as intense as that of oxychlorocruorin, but to be on the safe side no ratio is given as being over 3. The second error is in measuring the length of the worms, for they were alive and contractile; had time permitted dry weights would have been determined. The correlation coefficient, however, of the intensity ratio of the  $\alpha$ -bands on length of worm is  $-0.508$ , which, with 44 degrees of freedom, means a probability of less than 1 in 100 that the relationship found is due to chance alone.

TABLE 1. RATIO OF INTENSITY OF  $\alpha$ -OXYHAEMOGLOBIN TO  $\alpha$ -OXYCHLOROCRUORIN ABSORPTION BANDS IN THE BLOOD OF INDIVIDUAL *SERPULA VERMICULARIS* OF VARIOUS BODY LENGTHS

length (mm.)	60	50	26	22	21	21	20	20	20	19	19	18	17	17	17
ratio	1	1	0.75	0.75	1	2	0.5	1	2	0.5	2	2	1	2	2
length (mm.)	16	15	15	15	15	14	14	14	13	12	11	11	11		
ratio	2	0.75	1.25	2	2	1	2	2	1.25	1	2	2	3		
length (mm.)	10	10	10	10	9	9	9	9	8	8	8	7	7	7	6
ratio	1	2	2	3	1.5	1.5	3	3	2	2	3	2	3	3	2

MacMunn in 1885 saw, in the blood of *S. contortuplicata* (= *S. vermicularis*), the composite spectrum of oxychlorocruorin and oxyhaemoglobin, but he did not recognize it as such. Three of the spectra in his plate 33 show the mixed blood pigments in the living animals. In no. 11 the oxychlorocruorin and oxyhaemoglobin  $\alpha$ -bands are equal in intensity; in no. 7 the former and in no. 10 the latter is stronger.

Equal intensity, however, of the two  $\alpha$ -bands does not necessarily mean equal concentration of the pigments, since the  $\alpha$ -band of oxychlorocruorin absorbs more light than that of oxyhaemoglobin. Warburg (1932) gives the absorption constant,

\* *Serpula lobiancoi*, according to Fauvel (1927), is known only from the Atlantic coast of Spain. I found it to be not uncommon on stones in 40 to 50 m. of water at La Gaiola, Naples. It is a small species with a bilaterally symmetrical crown. My biggest specimen was 25 mm. long. The tube is pink as in *S. vermicularis*. Except for the anterior end, it is rolled in a close spiral, 8 mm. across. Fauvel states that the animal's coloration is unknown. The crowns of mine were all different, being largely colourless, with red, white and occasionally orange markings.



$K$ , of carboxychlorocruorin from *Sabella spallanzanii* at the wave-length  $600\text{ m}\mu$ , which is the axis of the  $\alpha$ -band, as  $0.45 \times 10^8$ . The absorption constant,  $K$ , is defined by the equation

$$K = \frac{1}{lc} \times \log_e \frac{I_0}{I},$$

where  $l$  is the thickness of the optical cell in cm.,  $c$  is the concentration in g. atoms Fe/ml., and  $I_0/I$  is the optical density, or ratio of the initial and final light intensities. I find that the ratio of the optical density of oxychlorocruorin to carboxychlorocruorin of the same concentration at the axes of their respective  $\alpha$ -bands is  $1.45/1.35 = 1.083$ . Therefore, for oxychlorocruorin at  $602\text{ m}\mu$  (the axis of the  $\alpha$ -band), Warburg's absorption constant\* becomes  $0.49 \times 10^8$ . The corresponding figure for oxyhaemoglobin at the wave-length  $578\text{ m}\mu$ , which is the axis of its  $\alpha$ -band, can be obtained from Vlès (1921), who gives his constant (specific extinction coefficient) in terms of concentration of haemoglobin and  $\log_{10}$ . Converting to Warburg's notation, we get  $K = 0.30 \times 10^8$ . Thus, for equal pigment concentrations, the  $\alpha$ -band absorptions of oxychlorocruorin and oxyhaemoglobin are as 49:30. From this it follows that equal density of the bands in two solutions would mean 1.63 times as much haemoglobin as chlorocruorin.

But in a mixture of oxychlorocruorin and oxyhaemoglobin, as found in *Serpula* blood, we must take into account the light absorption of each pigment at the  $\alpha$ -band of the other pigment. At  $580\text{ m}\mu$  Warburg's  $K$  for carboxychlorocruorin is  $0.28 \times 10^8$ . I have found that at this wave-length the ratio of light absorption by oxy- to carboxychlorocruorin is 65:75; therefore  $K$  for oxychlorocruorin is  $0.24 \times 10^8$ . Calculated from Vlès's data,  $K$  for oxyhaemoglobin at  $602\text{ m}\mu$  is  $0.02 \times 10^8$ . In a mixture, then, of oxychlorocruorin and oxyhaemoglobin of equal concentrations, the apparent intensities of the respective  $\alpha$ -bands will be as  $49 + 2:30 + 24$ , or 51:54. Therefore, with equal intensities of the  $\alpha$ -bands in *Serpula* blood, chlorocruorin and haemoglobin will be present in almost equal amounts. There is, however, probably a slight error in this deduction, due to the fact that chlorocruorin is not completely in the oxy state at atmospheric partial pressure of oxygen (Fox 1932), but the extent of this unsaturation is not known for *Serpula*.

The chlorocruorin of *Sabella* has a lower oxygen affinity than that of human haemoglobin. This has been shown quantitatively for *S. spallanzanii* (Fox 1932), the oxygen affinity of the chlorocruorin at  $17^\circ\text{C}$  being equal to that of human haemoglobin at  $37^\circ\text{C}$ , whereas at  $17^\circ\text{C}$  the human blood pigment has a much higher oxygen affinity. Other mammalian blood haemoglobins have oxygen affinities of the same order as that of man, and this is true too of various invertebrate blood haemoglobins (Fox 1945). But some fishes have haemoglobins with as low an oxygen affinity as the chlorocruorin of *S. spallanzanii* (Hall & McCutcheon 1938).

The low oxygen affinity of the chlorocruorin of *Sabella* was first shown qualitatively (Fox 1926) by making a mixture of *S. pavonina* and human bloods, sealing a few drops with a fragment of living tissue beneath a cover-slip in a glass micro-

\* The absorption constant for oxychlorocruorin is here taken indirectly from Warburg's data, not directly from those of Roche & Fox (1933), because there seems to be an error in the iron analysis of the latter.

scope cell and watching the fading of the  $\alpha$ -oxy-bands as the pigments are de-oxygenated. The oxychlorocruorin band goes before that of oxyhaemoglobin. I have now repeated the experiment with the same result on the diluted bloods of *Branchiommma vesiculosum*, *Myxicola infundibulum* and *Protula intestinum*, each mixed with my own haemoglobin; the result was the same as before, showing that the chlorocruorins of various origins (including one from a species of the Serpulidae) all have a low oxygen affinity. When, however, a corresponding experiment is made with the blood of *Serpula* alone, shutting it up with a fragment of living worm, the oxychlorocruorin and oxyhaemoglobin  $\alpha$ -bands fade at the same time. Here, then, the two pigments have the *same* oxygen affinity as one another. Evidently, either the chlorocruorin must have an unusually high oxygen affinity or the haemoglobin an unusually low one.

#### 4. TISSUE HAEM IN THE SERPULIMORPHA

Most animal cells contain haem compounds (Keilin 1929). In some the haems appear as cytochrome, with its characteristic absorption bands when in the reduced state. Other cells show no such bands but contain some protohaem, which is present in such small amounts that it can only be detected by converting it into a haemochromogen. Since haemochromogens have an intense  $\alpha$ -band, small quantities can thus be demonstrated. Pyridine is a convenient reagent for forming haemochromogens with haems in cells or body fluids. It gives a haemochromogen with protohaem, which has its  $\alpha$ -band at 557 m $\mu$ . Protohaem, detected thus, is found to be very widespread. It is now of particular interest to know whether animals with chlorocruorin in their blood have protohaem or chlorocruorohaem in their cells. This was investigated by treating various tissues of *Sabella spallanzanii* with pyridine and sodium hydrosulphite.

The pyridine haemochromogen of chlorocruorin in alkaline or weakly acid solution has a typical haemochromogen spectrum, with an  $\alpha$ -band at 583 m $\mu$  and a  $\beta$ -band at 540 m $\mu$ . In stronger acid, however, there is a spectrum with three bands in the visible region, at 583, 553 and 524 m $\mu$ . It is important to avoid this spectrum, because the band at 553 m $\mu$  could partially conceal a weak  $\alpha$ -band of pyridine-protohaemochromogen at 557 m $\mu$ . It can be avoided by using only a minimal quantity of hydrosulphite crystals.

The muscle of *S. spallanzanii* was first tested for haem. For this purpose it is essential to remove all adherent blood capillaries. The dorsal muscle was taken, since this has no capillaries inside it (Ewer 1941), and all capillaries were dissected from its inner face under a binocular microscope. The colour of the muscle is very light brown. I could detect no cytochrome\* in it, nor any haemochromogen by cutting it up with hydrosulphite. On the addition of pyridine, however, the protohaemochromogen band at 557 m $\mu$  appeared. There was no band at 583 m $\mu$ , so chlorocruorohaem is absent.

\* I found no cytochrome in any tissue of the Serpulimorpha. It is, however, present in the Annelida, for instance, in the gizzard of the earthworm *Allolobophora*, while the body wall and tentacles of *Amphitrite* have a single haemochromogen with its  $\alpha$ -band at 560 m $\mu$ .

The same result was obtained with the eggs of *S. spallanzanii*; they contain only protohaem. This was the case, too, with the eggs of *Protula intestinum*. From this animal, sperm was also tested. Undiluted sperm was obtained from males just removed from their tubes, when they spawned out of water. The sperm, too, showed only protohaem.

Several other tissues of *Sabella spallanzanii*, from which blood vessels could not be removed, were also tested with pyridine. These were the crown, the ventral gland shields, the gut wall and the thoracic nephridia. All of them showed both protohaem and chlorocruorohaem. The latter probably came from the blood, but one cannot say whether or not some of it may have been in the tissues. One can be sure, however, that none of the protohaem was derived from the blood, for blood alone with pyridine shows only chlorocruorohaem; it contains no protohaem. The crown consists largely of skeleton and blood vessels; its protohaem may well be in the former.

Chlorocruorin is thus apparently found only in the blood of the Serpulimorpha, not in cells. It was thought at one time that the coelomic fluid also contains the pigment (Romieu 1923). I was once rash enough to accept this statement without testing it, in attempting to explain the presence of chlorocruorin in the mucous tube of *Myxicola* (Fox 1926). I have now convinced myself that it is not true. It is difficult to open the body wall without puncturing any blood vessels, thus contaminating the body cavity fluid which exudes. This can be done, however, by making a dorsal posterior incision under a binocular microscope in a large *M. infundibulum* out of water. A fluid containing corpuscles and eggs or sperm exudes; it is quite devoid of chlorocruorin. Since chlorocruorin is thus absent from the coelomic fluid, and is only known to be present in the blood, the important question still remains to be answered as to where it is synthesized. The blood contains no corpuscles (Hanson 1949) and no heart-body.

*Potamilla*, with chlorocruorin in the blood, was found to differ from other Serpulimorpha in having haemoglobin in its muscles.\* In *P. reniformis* (O. F. Müller) the muscles are pink. If the abdomen is laid open, the blood vessels, green with chlorocruorin, form a striking contrast as they cross the muscles which are pink with haemoglobin. The  $\alpha$ -band of the latter is at 579 m $\mu$ . There is much less muscle haemoglobin in *P. stichophthalmus* (Grube). As the two species burrow side by side in the same stones, it is not clear why functionally they should have such different amounts of muscle haemoglobin, unless it is because *P. reniformis* is bigger. The possession of muscle haemoglobin by *Potamilla*, and of two blood pigments by *Serpula*, are new chemical generic characters to be commended to the taxonomists. The eggs of *P. reniformis* contain no haemoglobin; treated with sodium hydrosulphite they show no haemochromogen band. But on the further addition of pyridine they reveal an astonishing concentration of protohaem.

\* I purposely write 'haemoglobin', not 'myoglobin', for if we are to use the latter term for haemoglobin in muscle, we should have other words for haemoglobin in nerve ganglia and in glands, where it occurs in some invertebrates. Similarly, I avoid complicating terminology by using 'erythrocrucorin' for the haemoglobin of invertebrates. This old name has been revived for invertebrate haemoglobins with different amino-acid proportions and a different isoelectric point from the haemoglobins of gnathostome vertebrates. But we do not know whether or not all invertebrate haemoglobins have these properties.

*Potamilla* is not the only annelid with muscle haemoglobin. It has long been known that the pigment is present in the pharyngeal muscles of *Aphrodite* (Lankester 1872). I find it present in the body-wall muscles of *Arenicola*, but absent from those of *Amphitrite* and *Allolobophora*. *Thalassema*, however, has haemoglobin in its body wall.

#### 5. THE EXCRETION OF HAEM

Haem is present in the mucoprotein tube of *Sabella*. Treatment with pyridine shows the protohaemochromogen  $\alpha$ -band at 557  $m\mu$ . There is more haem in the inner layers of the main part of the tube than in the outer layers or in the posterior pink part. There is likewise protohaem in the membranous lining of the calcareous tube of *Protula*. I know of no other case of the secretion or excretion of haem as such—not broken down to bile pigment or porphyrin—except chlorocruorin in the mucous tube of *Myxicola* (Fox 1926), haemochromogens in the gut lumen of various invertebrates and in egg vesicles of *Planorbis*, and the occasional presumably pathological presence of haemoglobin in the excretory organs of *Daphnia* (Fox 1948b).

Haemochromogens have been found in solution in the gut contents of decapod Crustacea (MacMunn 1883), phyllopod Crustacea (Fox 1948b), and pulmonate Mollusca (Dhéré & Vegezzi 1917). I find the haemochromogen  $\alpha$ -band at 561  $m\mu$  in *Astacus*, at 563  $m\mu$  in *Daphnia*, while in *Helix pomatia* L. (helicorubin) it is double, at 563 and 558  $m\mu$ . In the Serpulidae there is also a gut haemochromogen, which has its  $\alpha$ -band at 560  $m\mu$ . It can be seen with the microspectroscope in a small animal held in a compressorium. The haemochromogen is usually in the reduced state, but the intensity of the band may be increased by sodium hydrosulphite in the water outside the animal. The concentration of this haemochromogen varies with individuals. It can be high in the anterior third of the abdomen, where there is a swelling of the intestine, in *Serpula vermicularis* L., *Vermiliopsis infundibulum* (Phillipi), *Protula intestinum* L. and *Apomatus similis* Marion. In *Hydroides norvegicus* (Gunnerus) and *H. uncinata* (Phillipi) the band is visible in thorax and intestine. I was able to extract the gut liquid from *Apomatus similis* by puncturing the abdomen of a specimen out of water. The drop of orange liquid was put in a compressorium and its thickness increased by screwing up the cover-slip. The strong  $\alpha$ -band at 560  $m\mu$  and a weak  $\beta$ -band were seen, showing that the haemochromogen is indeed in solution in the gut liquid. Pyridine added to this drop moved the  $\alpha$ -band to 557  $m\mu$ , so that it is a protohaem compound. From a large *Protula intestinum* brown gut liquid containing the haemochromogen was also extracted, and some of the gut wall was dissected out and found to be devoid of the pigment. *Myxicola aesthetica* (Claparède), too, has the haemochromogen in its gut, but I have been unable to find it in any species of the Sabellidae.

The Serpulimorpha are not the only Annelida with a haemochromogen in the gut. The liquid extracted from the intestine of *Polycirrus calendrium* Claparède showed at first no bands, but on the addition of hydrosulphite the haemochromogen bands appeared, with  $\alpha$  at 560  $m\mu$ . It might be thought that this wave-length is characteristic of the Polychaeta, but this is not so. The oesophageal pouches of *Arenicola*

*marina* (L.) contain a high concentration of a haemochromogen with an  $\alpha$ -band at 563 m $\mu$ . This is the same wave-length as in *Daphnia*.

It is unknown whether these intestinal haemochromogens are excretory products, or whether they are accumulated from haem in the food. If excretory, the substance must, in the serpulids, be derived from the tissue protohaem. This leaves the question unanswered as to what, if any, is the excretory product of chlorocruorin.

The major part of this investigation was made at the Zoological Station of Naples, in 1947. Details were added from living material sent from Plymouth to London. The work on carbon monoxide was done at the Molteno Institute, Cambridge. I am greatly indebted to Dr R. Dohrn and Professor D. Keilin for their welcome and help, as well as to the staffs of the Stazione Zoologica and the Molteno Institute. Preliminary accounts of parts of the work have been published (Fox 1947, 1948a).

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## Fluctuations in the haemoglobin content of *Daphnia*

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Pond-dwelling species of *Daphnia* are known to synthesize blood haemoglobin in poorly aerated natural waters. It has now been found that a lake plankton species, the blood of which is colourless in nature, also synthesizes haemoglobin in artificial conditions of oxygen deficit.

Haemoglobin formation in *Daphnia*, resulting from a low oxygen content of the water, increases with the amount of algal food eaten, up to a certain nutritional level. Chlorophyll in the food has not been found to favour haemoglobin synthesis.

In the water of ponds harbouring red *Daphnia* we have found no factor stimulating haemoglobin synthesis other than oxygen deficit.

The haemoglobin content of the blood of *Daphnia* varies within each instar. It is least when eggs are laid by the parthenogenetic female in her brood pouch and greatest when the eggs have developed into late-stage embryos ready to be released. After this, during the few hours before the moult and the laying of more eggs, the haemoglobin content of the blood decreases rapidly. At the same time the ovaries gain haemoglobin. This implies that haemoglobin passes from the blood into the ovaries shortly before the eggs are laid. During the development of the eggs in the brood pouch, the blood of the female gradually reacquires its full haemoglobin content.

### 1. INTRODUCTION

In 1758 Linnaeus wrote of *Daphnia*, which he called *Monoculus Pulex*: 'Habitat ubique in aquis dulcibus, tanta saepe in copia, ut appareat sanguinea.' This is a good description, but *Daphnia pulex*, when abundant in ponds or ditches, does not by any means always colour the water like blood. The population may be colourless or merely pink. The intensity of the red colour, which is due to haemoglobin in solution in the blood of *Daphnia*, varies inversely with the oxygen content of the water (Fox 1948).

This has been shown to be so in the three common British species of pond dwellers, *D. magna* Straus, *D. pulex* (De Geer) and *D. obtusa* Kurz. But *D. hyalina*

Leydig, living in lake plankton, has blood that is colourless. Nevertheless, not only does it contain traces of haemoglobin, but in artificial conditions of oxygen deficiency enough haemoglobin is synthesized to make the animals pink. This was seen at Pallanza in Italy, where, in the garden of the Hydrobiological Institute, there are two large cement experimental tanks containing water from the Lago Maggiore. The water of one of the tanks had been enriched with fertilizer (ammonium sulphate and 'superphosphate of lime'). In August both tanks contained abundant *D. hyalina*. In the untreated tank of clear water the cladocerans were as colourless as in the lake. Yet when a mass of them was squashed in muslin and the exuded liquid examined with a spectroscope, the bands of oxyhaemoglobin were clearly seen. In the fertilized tank the cladocerans varied in colour from week to week, between pink and colourless, in relation to the oxygen deficit of the water. Occasionally this water was less than 50 % air saturated owing to abundant micro-organisms, and then the *D. hyalina* was pink. Usually the water was supersaturated with oxygen at the atmospheric partial pressure, owing to photosynthesis by abundant *Microcystis*, and then the cladocerans were colourless.

Thus an increase in haemoglobin in response to oxygen lack is apparently a general property of the blood of *Daphnia*, even of species which in nature have blood that is quite colourless to the eye. Perhaps this faculty is common to most Entomostraca with haemoglobin in their blood, for it is very marked in *Apus*, *Triops cancriformis* (Bosc.). In nature the colour of this animal is usually described as dull green or brown. This is its aspect when grown in the laboratory in a flat dish of very shallow water. But if cultured in a deep vessel with bacterial mud on the bottom, which reduces the oxygen content of the water to low values, *Apus* becomes bright red from much haemoglobin in the blood (Fox 1949).

The work reported below was begun in order to find out if there are factors in pond water other than oxygen deficiency which stimulate haemoglobin synthesis in the blood of *Daphnia*. In the course of this investigation, in which parthenogenetic females were used, the discovery was made of the passage of haemoglobin from the blood into the eggs.

## 2. METHODS

The following species were studied: *Daphnia obtusa*, *D. pulex* and *D. magna*.

The concentration of haemoglobin in the blood was measured by the method of Fox (1948). The pink colour of a known dilution of the observer's blood in a glass wedge trough, the image of which is projected on to part of the microscope field, is compared with the blood colour of individual *Daphnia* seen in the remaining part of the field. We now find it useful to distinguish between the terms haemoglobin *value* of one individual and haemoglobin *index* of a population, i.e. the mean of the values for ten large individuals. A value or index of over 100 denotes very red *Daphnia*, whereas blood with a value below 15 is too pale to be measured.

The reliability of the index as a measure of the haemoglobin concentration in the blood of a population is given by the standard error of the index. The variation of the index measured by the standard error arises from two main sources: from the variation between individual animals in the sample and from the experimental

errors in determining the haemoglobin value of each animal, such as the visual errors of colorimetry. The mean standard error of 151 separate index determinations, over a wide range of indices from 24 to 95, was found to be 3.7 % of the index. In order to determine what proportion of the standard error of the index is due to variation in haemoglobin content of the animals and what is due to experimental factors, the following test was made. Ten *D. magna* were taken from one population, and the haemoglobin value of each animal was determined 10 times in the course of a day. Males and immature parthenogenetic females were used, so that there might be no change in the blood haemoglobin values of individuals in the course of the day due to egg-laying (see § 4 below). The haemoglobin value was first determined for each of the ten individuals in turn, and then this was repeated until ten determinations of each had been made. A two-factor analysis of variance showed that there was no significant trend in the errors of successive determinations on the same animal in the course of the day. The mean values for the ten animals gave, of course, ten measures of the population index. The variance in the population index due to difference between the animals was found to be 13.8, which was significantly much greater than the variance due to experimental errors, namely, 2.2. From this it is clear that experimental errors contribute little to the variability expressed by the standard error of the index.

After determining a haemoglobin index, the mean size of the individuals in the population was estimated by measuring with an eyepiece micrometer the length of each of the ten animals, from forehead to base of posterior shell spine. The parthenogenetic young in each brood pouch were then dissected out and their mean number found. It is known that not only the size of *Daphnia* but also the number of eggs laid in the brood pouch is dependent upon nutrition (Ingle 1933; Fox 1948, p. 202); in captivity an increase in quantity of food increases the number of young produced. Therefore the mean number of eggs or embryos was used by us as a measure of nutrition.

The number of eggs or embryos carried by a female indicates the level of nutrition during the preceding instar. This is so because the eggs which are formed in the ovaries during one instar are laid in the brood pouch after a moult has taken place, and thus the effect of nutrition in one instar is not revealed until the following instar. Our experiments were sufficiently long for the influence of feeding to show itself by the number of eggs or young in the brood pouch.

Dissolved oxygen was measured by the syringe-pipette modification of the Winkler method (Fox & Wingfield 1938). Some pond waters contain nitrite. This falsifies the Winkler method by liberating iodine from iodide in acid solution. The presence of nitrite can be revealed by taking pond water into the pipette without manganese chloride and then taking in the other Winkler reagents. Starch shows if any iodine has been liberated. If so, the dead space of the syringe-pipette is filled with sodium azide solution before the analysis, the water sample being then taken in, and the manganese chloride after it.

Most experiments consisted in comparing the haemoglobin production of *Daphnia* under various conditions, e.g. different waters or different foods. The experiments lasted a week or more. When possible they were made in the animals' own filtered



pond water. If a low oxygen content of the water was required, a series of 250 ml. flasks was used under each of the experimental conditions. Initially the oxygen concentration was reduced by bubbling nitrogen through the water. The number of *Daphnia* was proportional to the water volume, namely, one *D. pulex* or *D. obtusa* to 2 ml. of water. The amount of algal food was also proportional to the water volume. The flasks were kept in the dark to prevent oxygen production through photosynthesis. When an experiment lasted longer than a week, the water was changed half-way through the experiment. Respiration of *Daphnia* and algae kept the oxygen content of the water low, and the small surface prevented much diffusion of air into the water. Since the flasks were filled to various levels in the neck, a range of oxygen contents resulted. Oxygen analyses were usually made every second day. Out of the series of flasks, a number would be found with similar steady low oxygen contents under each of the experimental conditions. In the tables below, the mean percentage air saturation of each of these flasks during the experiment is given; 50 % air saturation, for example, means a dissolved oxygen content of the water of 3.35 ml./l. at 18° C. The experiments were done at room temperature, which, of course, was variable; constancy was unnecessary, since in each experiment the several flasks were kept at the same room temperatures.

The following organisms were used as food: *Chlorella vulgaris* Beij, *Gonium pectorale* Müller, *Chlamydomonas incisa* (Pringsheim), and *Polytoma uvella* Ehrenberg. The algae, both green and colourless, were cultured by standard methods. For feeding *Daphnia*, algae were centrifuged from measured volumes of the liquid culture medium, or of a water suspension of those grown on agar, and then resuspended in the water of the culture flasks. This method of feeding does not appreciably alter the experimental conditions in the flasks. For approximately constant feeding with *Chlorella* an artificial colour standard was made up of potassium dichromate, nickel sulphate and kieselguhr (for opacity). The *Chlorella* suspension was matched against this standard, and thus comparable feeding conditions were obtained in consecutive experiments. For precise equality of feeding with different algae, the method described on p. 392 was used. Preliminary tests showed that daily addition of food maintained *Daphnia* in better condition than giving a large quantity of algae at the beginning of an experiment only, or feeding on every second day.

### 3. FACTORS IN HAEMOGLOBIN SYNTHESIS

The haemoglobin content of the blood of *Daphnia* increases when the dissolved oxygen content of the water falls low. What other factors are involved in this haemoglobin synthesis?

#### (a) Nutrition

The question of nutrition was first studied. It was not improbable that a minimal nutritional level would be required for haemoglobin to be produced as a result of oxygen deficiency. One experiment may be quoted out of a number which showed this to be the case. Pale *D. pulex* with a haemoglobin index of 25 was kept for a week in water of low oxygen content (a) without food, and (b) fed daily on *Gonium*. In the two foodless flasks the water was 10 and 11 % air saturated and the final

haemoglobin indices were 27 and 30, i.e. they had hardly risen. In both of the flasks with food the water was 9% air saturated (virtually the same as in the foodless flasks) and the indices had risen to 46 and 56. The starved animals carried no young, while in the flasks with food the mean numbers of young were 3.1 and 1.9.

The next question regarding feeding was a quantitative one. Is the haemoglobin production at a low oxygen content of the water proportional to the amount of food given? Table 1 summarizes one of the experiments made to test this matter. Pale *D. obtusa* was kept for 13 days in water of low oxygen content, and fed for the first 8 days on *Gonium*, then on *Chlorella*. Food was added daily to different flasks in amounts proportional to 1:2:4:8. Table 1 shows that, as regards the production of young, the optimum food concentration was reached at food value 4. Haemoglobin production was proportional to the quantities of food for values 1, 2 and 4, but hardly any more haemoglobin was produced at 8 than at 4.

TABLE 1. THE EFFECT OF DIFFERENT AMOUNTS OF ALGAL FOOD ON HAEMOGLOBIN PRODUCTION IN 13 DAYS BY PALE *DAPHNIA OBTUSA* (INDEX: 21) IN WATER OF LOW OXYGEN CONTENT. Initial mean number of young: 0.2

food ratio ...	1			2		4			8		
flask no. ...	1	2	3	4	5	6	7	8	9	10	11
haemoglobin index, last day	52	56	59	58	65	83	74	78	81	82	84
mean % air saturation	23	9	8	12	7	12	7	7	9	6	6
mean no. of young, last day	2.3	2.0	2.3	5.4	8.8	13.5	8.7	8.7	8.9	7.1	7.2

Other experiments led to the same conclusion, namely, that haemoglobin synthesis at low oxygen contents of the water requires a minimal nutrition and increases with the amount of food up to an optimal value of the latter. This may be the explanation of the apparent stimulating effect of duck faeces on haemoglobin formation by *Daphnia* (Fox 1948). At the time when those experiments were made no check was kept on nutrition by counts of the number of young, and the faeces may well have supplied a richer bacterial pabulum.

#### (b) *Chlorophyll*

In the past, chlorophyll in food has been thought to be responsible for haemoglobin synthesis in *Daphnia* (Verne 1923). Qualitative experiments led to the conclusion that this is not true (Fox 1948), but owing to similarities in the chemical structure of chlorophyll and haem the implications are so important that we thought it worth while to make quantitative tests.

The most conclusive experiment is summarized in table 2. The green *Chlamydomonas incisa* and the non-green *Polytoma uvella*, both members of the same family of the Volvocales, were used as food. In this experiment precisely equal amounts of the two organisms were given daily to *D. obtusa* during the week of the experiment, which again was made in water of low oxygen content. *Chlamydomonas incisa* is larger than *Polytoma uvella*. In order to overcome this difference in size,

the relative weights of the two organisms were determined. Known numbers of cells of each organism in suspension in water, found with the aid of a haemocytometer, were centrifuged, dried and weighed. It was found that the weight of one *Chlamydomonas incisa* is 5.3 times the weight of one *Polytoma uvella*. For feeding, suspensions of the organisms were made each day, and the number of cells in each suspension was found by using the haemocytometer. The requisite volume of each suspension required for equal weight of food was then calculated. Table 2 shows, from the numbers of eggs produced, that the two foods were equally nutritious. Furthermore, like amounts of haemoglobin were formed with the green and non-green foods, amounts which were in inverse proportion to the oxygen contents of the waters. Thus there is no indication of a favourable influence of chlorophyll on haemoglobin formation, at least during the time of the experiment, namely, a week.

TABLE 2. THE EFFECTS OF FEEDING WITH EQUAL AMOUNTS OF A GREEN AND A COLOURLESS ALGA ON THE HAEMOGLOBIN PRODUCTION OF PALE *DAPHNIA* *OBTUS* (INDEX: 28) KEPT FOR A WEEK IN WATER OF LOW OXYGEN CONTENT

food	...	<i>Chlamydomonas</i>			<i>Polytoma</i>		
flask no.	...	1	2	3	4	5	6
haemoglobin index, last day		36	45	56	38	53	56
mean % air saturation		27	12	6	28	12	9
mean no. of young, last day		3.3	3.5	2.8	2.3	3.9	3.1

(c) Pond water

Preliminary work (Fox 1948) had indicated that the haemoglobin content of pale *Daphnia* kept in the laboratory will increase in water taken from a pond harbouring red *Daphnia*, even when the oxygen content of the water is not low. This suggests the presence of some substance in such waters stimulating haemoglobin synthesis. Experiments were accordingly made to find out whether such a substance can be found.

An attempt was first made to separate the influence of oxygen deficiency in the water on the haemoglobin content of *Daphnia* blood from that of a possible other factor in the pond water. With this in view, experiments were made in air-saturated water, but no significant rise in the haemoglobin index of pale *Daphnia* was obtained in water from ponds which contained red *Daphnia*. Further experiments were therefore made at low levels of oxygen concentration.

TABLE 3. THE HAEMOGLOBIN CONTENT OF ORIGINALLY PALE *DAPHNIA* *PULEX* (INDEX: 25) KEPT FOR A WEEK IN ITS OWN POND WATER AND IN WATER FROM ANOTHER POND WHICH IN NATURE CONTAINED RED *D. PULEX* (INDEX: 110).

water	...	own pond				another pond		
flask no.	...	1	2	3	4	5	6	7
haemoglobin index, last day		43	42	46	56	33	32	38
mean % air saturation		18	15	9	9	13	12	12
mean no. of young, last day		2.0	1.1	3.1	1.9	3.5	1.7	2.5

The results of one of these experiments are given in table 3. The water tested came from a pond containing red *D. pulex* with a haemoglobin index of 110. The experimental animals were pale *D. pulex*, which in their own pond had an index of 25. They were kept for a week (*a*) in their own pond water, and (*b*) in the water from the pond containing red *Daphnia*. The waters were filtered and had a low oxygen content. *Gonium* was given daily as food. Table 3 shows that the haemoglobin index rose in all of the experimental flasks as a result of the low oxygen content of the water. It rose most when the oxygen was lowest. But the index rose more in the animals' own pond water than in the other water, although the state of nutrition, judged by the number of young, was, if anything, better in the latter than in the former. This and other experiments gave no indication that water from ponds containing red *Daphnia* favours haemoglobin synthesis.

The result of the preliminary experiment quoted above (Fox 1948) seems to have been due to good nutrition. It is our experience that with sufficiently abundant food some haemoglobin may be synthesized as a result of an oxygen deficit of as little as 10 to 20 % below the value at air saturation.

#### 4. PASSAGE OF HAEMOGLOBIN FROM BLOOD INTO EGGS

##### (*a*) *Loss of blood haemoglobin previous to egg laying*

The haemoglobin content of the blood of *Daphnia* does not remain constant, even with a steady low oxygen content of the water and adequate nutrition. Parthenogenetic females carrying embryos in the brood pouch tend to have more haemoglobin in the blood than those with eggs in the pouch. The differences are considerable. For example, in the eight flasks of a certain experiment on haemoglobin production by *D. obtusa*, in waters of various low oxygen contents, the haemoglobin indices for females carrying embryos were 71, 72, 72, 76, 78, 84, 85, 86, while the corresponding indices for those with eggs in the pouch were 51, 46, 53, 52, 51, 60, 60, 54. The average index of females carrying eggs was thus only about two-thirds of the index of females carrying embryos.

The eggs of *Daphnia* are known to contain haemoglobin (Teissier 1932), and the simplest explanation of the lower haemoglobin content of the blood of females with eggs in the brood pouch compared with those carrying embryos is that the pigment passes from the blood into the developing oocytes in the ovary before the eggs are laid, and that as the embryos develop in the brood pouch the haemoglobin of the female's blood is regenerated. Table 4 gives data from eleven wild populations. The table shows that the greatest difference in haemoglobin index corresponds to the greatest number of young in the brood pouch, and the least difference to the fewest young, which supports the hypothesis that the eggs receive their haemoglobin from the blood. There is a significant positive correlation of the difference in index between the egg-carrying females and the embryo-carrying females quoted in table 4 and the mean number of young of these females. The correlation coefficient is +0.574, corresponding to a probability (for 9 degrees of freedom) of only 1 in 18 that the result is due to chance alone.

TABLE 4. HAEMOGLOBIN INDEX OF THE BLOOD OF FEMALES CARRYING EGGS AND OF THOSE CARRYING LATE EMBRYOS, IN ELEVEN WILD POPULATIONS OF *DAPHNIA*. Each population consisted of one species only of *D. magna*, *D. pulex* or *D. obtusa*. In each population the haemoglobin values of ten females with eggs and ten with embryos were measured to give the two indices.

population no.	haemoglobin index			mean numbers of	
	with embryos	with eggs	% difference	embryos	eggs
1	44	25	42	37	34
2	88	60	32	19	20
3	86	59	32	19	18
4	50	35	30	14	16
5	57	41	27	14	11
6	59	43	27	11	17
7	51	38	26	13	17
8	91	68	25	19	26
9	85	68	20	5	8
10	92	86	7	11	4
11	85	83	2	7	4

*Daphnia* lays eggs in the brood pouch about half an hour after moulting. It was found that a dramatic drop in the haemoglobin index begins a few hours before the moult. Figure 1 gives the blood haemoglobin values of eleven individual *D. obtusa* at intervals over a period of from 6 hr. before moulting to 3 hr. after moulting. Each individual laid eggs after the moult. The loss of haemoglobin from the blood is seen to be at first gradual and then more rapid until the moult occurs. Control haemoglobin determinations were made on ten individuals at intervals over a period

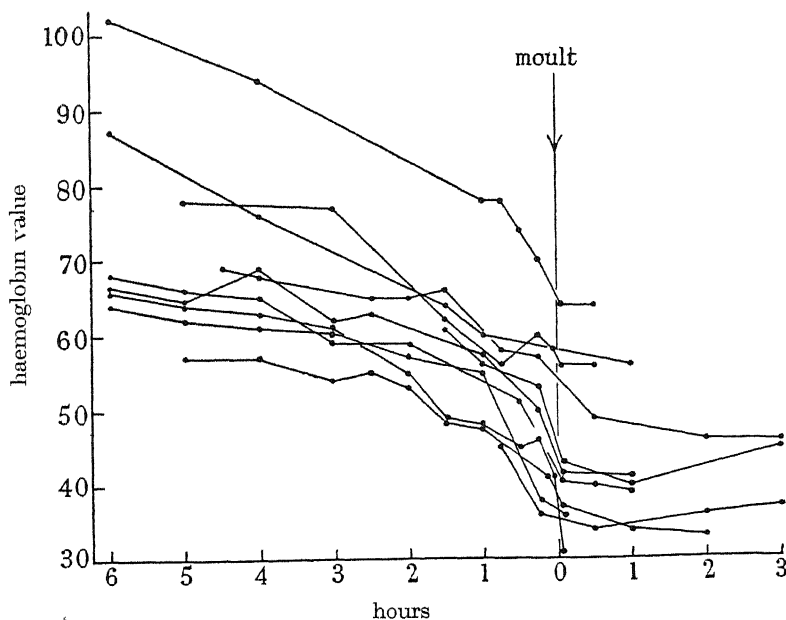


FIGURE 1. Haemoglobin values of eleven individual *Daphnia obtusa* before and after moulting.

of 7 hr. not preceding a moult to be sure that the drop in haemoglobin in figure 1 was not just due to the high oxygen content of the water. There was no drop in the control animals: indeed, the time was clearly too short for such a considerable loss of haemoglobin to occur.

When *Daphnia* moults there must be an apparent loss of blood haemoglobin owing to the suddenly increased size of the animal, with concomitant dilution of the blood. For the eleven animals of figure 1 the mean length (with its standard error) after moulting was  $1.11 \pm 0.02$  times that before. But the increased size after moult could not explain the considerable diminution of blood haemoglobin during the several hours previous to the moult.

#### (b) *Haemoglobin in ovary and eggs*

The eggs of *Daphnia* are laid from the two ovaries into the brood pouch. During the first hour after they are laid, the parthenogenetic eggs swell very considerably (Ramult 1914). While they are still small they appear dark grey, and then, on swelling, they may assume one of various tints of green or blue-green. This colour is due to carotenoid-protein compounds derived from carotenoids in the algal food (Teissier 1932). Or the eggs may lack these pigments, in which case the pink tint of their contained haemoglobin may be visible.

The ovaries, just after eggs have been laid, are small and colourless. As the ovaries gradually grow again they become grey, and later become greenish. During the few hours before eggs are again laid, the ovaries, now large, can be seen under the microscope by reflected light to become progressively pinker. In those oocytes which contain a green pigment, the reddish colour is particularly marked towards the periphery. Apparently the oocytes have acquired haemoglobin. That this is really the case was shown as follows. When large pinkish ovaries were dissected out of the body of *D. magna* into crayfish Ringer solution (Pantin 1946), the microspectroscope showed strong bands of oxyhaemoglobin. Younger ovaries, whose green oocytes had not yet acquired any pink colour, had much weaker oxyhaemoglobin bands. Small green ovaries, dissected out at a yet earlier stage of growth, showed no oxyhaemoglobin bands. Thus, as the ovaries grow again after each deposition of eggs, they gradually acquire haemoglobin. The very considerable increase in haemoglobin content which causes a visible reddening of the oocytes begins about 6 hr. before the next egg-laying; that is, it begins just at the time when the haemoglobin content of the blood commences to decrease.

The increase in the haemoglobin content of the ovaries coincident with a decrease in blood haemoglobin suggests a passage of unmodified protein across the cell membrane. It might be, however, that the blood haemoglobin is first broken down and then the products are resynthesized in the ovary. Whichever alternative is true, a passage of unmodified protein from blood into eggs would not be unique, for Wigglesworth (1943) found that in the louse (*Pediculus*) haemoglobin, after being absorbed from the food through the gut wall into the blood, penetrates into the eggs. Similarly, he found that in the blood-sucking bug *Rhodnius* the haemoglobin of the food appears as parahaematin (kathaemoglobin) in the blood, and the absorption spectrum of this pigment can be observed unaltered in the eggs.

A visible reddening of the oocytes as they develop in the ovary can also be seen. Another example of the uptake of unmodified protein by oocytes is found in the acquisition of passive immunity by birds' eggs. Here an antitoxin passes from the blood serum of the bird into the eggs (Fraser, Jukes, Branion & Halpern 1934; Andrewes 1939).

(c) *Regeneration of blood haemoglobin after egg-laying*

The time relations of the regeneration of haemoglobin in the blood after egg-laying were next studied. It is not feasible to measure the blood haemoglobin values of individual *Daphnia* at intervals during the period of a whole instar, lasting several days, since in that time the amount of haemoglobin would probably vary in response to changing oxygen content of the water. Another method had therefore to be adopted. The parthenogenetic eggs of *Daphnia* are laid in the mother's brood pouch shortly after a moult, and the embryos develop in the brood pouch until they are released to swim away; this occurs only a few hours before the next moult. It is therefore possible to assess the length of time that has elapsed since a moult by observing the stage of development of the embryos in the brood pouch. With this in view, the rate of development of the embryos was determined. A number of easily recognizable embryonic stages was selected, and the time intervals at 22° C between each stage and the next one were found.

Three populations of *D. obtusa* were then studied to find the time relations for the regeneration of blood haemoglobin. From each population six or seven lots of females were isolated, each lot having embryos at the same developmental stage. The six or seven stages were chosen so that the time intervals between each stage and the next were nearly equal. For each lot the haemoglobin index was determined. In this way each population could be studied in a single daytime, during which short time the haemoglobin values of the individuals should not alter appreciably owing to oxygen changes in the water.

The results are plotted in figure 2. The time scale for stages in the instar, corresponding to embryonic developmental stages, is that found previously for 22° C,

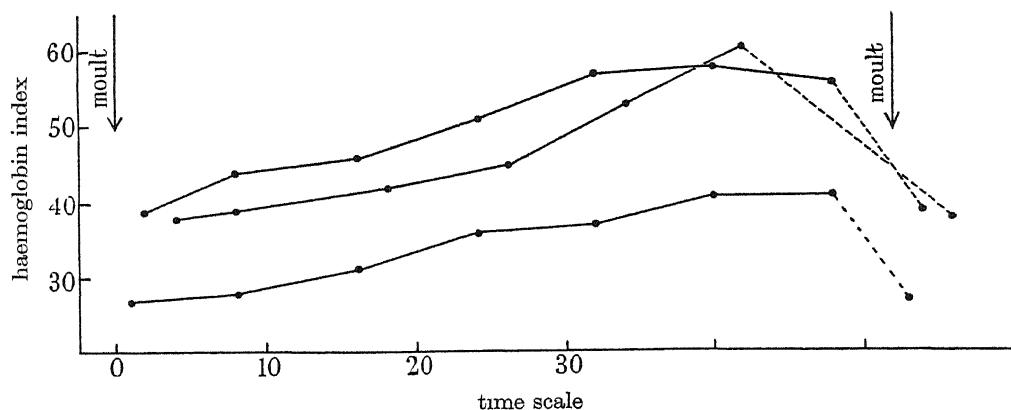


FIGURE 2. Haemoglobin indices of three populations of *Daphnia obtusa* between one moult and the next.

when an instar lasts approximately 52 hr. The three populations were studied at somewhat lower temperatures and the instars were correspondingly longer, but it was assumed that the ratios of the time intervals between the stages were unchanged. The dotted lines are drawn on the assumption that at the second moult the index returns to the value it had at the previous moult. It is clear from the figure that after a moult there is a gradual increase in the haemoglobin content of the blood until it reaches a maximum. The maximum is attained when there are late-stage embryos in the female's brood pouch. After this the haemoglobin content of the blood declines rapidly to reach its lowest value at the time of the next moult.

(d) *Modification of the index method*

The greatest concentration of haemoglobin is in the blood of females carrying late embryos. In consequence of this, a higher index figure for a population can be obtained by selecting adult females carrying late stage embryos rather than females taken at random. Moreover, the former procedure should result in a lower variance of the ten individual values from which the index is derived. For certain purposes, for example, for studying small increases in blood haemoglobin, such a higher index with a lower standard error can be of value. The reality of the higher index and lower variance was proved by the following analysis of thirty-four populations, in seventeen of which the indices were obtained from random females and in seventeen others from carriers of late embryos. In the two sets of seventeen populations the ranges of index values (from 27 to 93 and from 29 to 87 respectively) and the mean numbers of young (7 to 39 and 7 to 40) were virtually the same. For each set of seventeen populations the mean index and the mean standard error were calculated; for random selection the mean index was 47.42 and the mean standard error 2.54, while for late-embryo carriers the figures were 51.71 and 1.59. The variance ratio for the two mean standard errors is 2.56. For  $9 \times 17 = 153$  degrees of freedom this ratio has a probability of less than 1 in 1000 that the difference between the two mean index values is due to chance alone.

Most of the work reported in this paper was done in the Zoology Department of Bedford College. The observations on haemoglobin in *Daphnia hyalina* were made at the Istituto Italiano di Idrobiologia, Pallanza, to the Director of which, Professor Edgardo Baldi, warm thanks are due for his hospitality. We are grateful to Dr R. J. Whitney for statistical help. A preliminary note on part of the work has been published (Dresel 1948).

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## The abrupt transition from rest to activity in muscle

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The internal mechanical condition of an excited muscle has been examined by applying quick stretches at various moments after a maximal shock. At the end of the latent period there is an abrupt change of state, the contractile component suddenly becoming capable of bearing a load equal to the maximum tension set up in an isometric tetanus. The intensity of the active state produced by a shock is greatest at the start, is maintained for a time and then declines as relaxation sets in. The properties of the fully active state are defined by the three constants of the characteristic equation relating speed of shortening to load.

A muscle consists mechanically of three components: (1) a contractile one, (2) an undamped series elastic one and (3) a parallel elastic one. The complication provided by (3) is avoided by working with small initial loads. The load-extension relation of the series elastic component has been determined. Its extensibility is high at small loads, becoming much less at greater ones. The full isometric force produces an extension in it of about 10% of the muscle's length.

In an isometric tetanus the form of the myogram is fully determined by the characteristic force-velocity relation of the contractile component and the load-extension curve of the series elastic one, the former having to shorten and stretch the latter before an external tension can be manifested. In a twitch there is insufficient time, before relaxation sets in, for the full tension to be developed.

When the tension is raised sufficiently by a quick stretch applied early after a shock the contractile component cannot shorten as it would normally and the heat of shortening is absent. The heat of activation is probably a by-product of the process by which the sudden change of state from rest to full activity occurs.

When a muscle is subjected to a tension rather greater than it can bear it lengthens slowly; to a tension considerably greater it 'gives' or 'slips'. When a muscle is stretched rapidly a transitory overshoot of tension occurs followed by 'slip'. During the disappearance of this extra tension heat is produced, as in the 'cold drawing' of a wire or thread. An analogous process occurs in relaxation under a load.

When two shocks are applied in succession, the second restores the active state to its full intensity, from which it has declined to an extent depending on the interval after the first one. If, under isometric conditions, the series elastic component is still partly stretched at the moment when the second response occurs, the total tension developed is greater. This is the origin of the so-called 'supernormal phase' and the basis of the greater tension maintained in a tetanic contraction. During a tetanus each shock restores the active state of the muscle to its full intensity.

It seems reasonably certain that excitation of a muscle fibre occurs at its surface. It has been suggested that contraction is set up inside by the arrival of some chemical substance diffusing inwards after liberation at the surface. The onset, however, of full activity occurs so soon after a shock that diffusion is far too slow to account for it. A process, not a substance, must carry activation inwards.

## INTRODUCTION

The mechanical properties of striated muscle can be described in terms of three constituents: (1) the main contractile portion, (2) a passive elastic component in series with the contractile one, and (3) a passive elastic component in parallel with (1) and (2) (or, strictly speaking, passive elastic components in parallel with (1) *and* with (1) and (2)). It is not necessary for the following argument to identify constituents (2) and (3) precisely. Tendons and tendon bundles are certainly in series with the contractile portion, and the latter itself during activity probably enjoys elastic properties; while areolar and reticular tissue between the fibres, epi- and perimysium, and sarcolemma (Ramsay & Street 1940), must provide a parallel elasticity. At rest the contractile portion is highly extensible, and if muscle is stretched beyond the natural length which it occupies in the body the load is borne mainly by the parallel elastic constituent. It is possible, however, to study the chief properties of active muscle over a range of lengths at which, at rest, there is very little tension; it is simpler, therefore, to work within that range and to consider a system in which contractile and elastic elements in series with one another alone operate.

It is unusual for natural fibres to have a normal elasticity; with small loads the extensibility, or compliance, is high, but it diminishes rapidly with increasing load. It is not possible in a muscle at rest to examine the stress-strain relation of the series elastic elements; the contractile component is so extensible that by stretching a resting muscle the relation obtained between load and extension is mainly due to the parallel elastic body. It is possible, however, either from the form of the isometric myogram, or from the effect of quick releases during an isometric contraction, to deduce the stress-strain curve of the series elastic component alone. It shows the expected characteristics, the extensibility decreasing many times over the range from zero tension up to that exerted in a maximal isometric contraction, the total extension in that range being of the order of 10 % of the muscle's length.

The most fundamental mechanical property of the contractile portion of a muscle during activity is the characteristic relation between speed of shortening  $v$  and load  $P$ . This relation exists as an experimental fact independent of any equation to describe it; but in reality the 'characteristic equation' (Hill 1938; Katz 1939; Wilkie 1949)

$$(P+a)(v+b) = (P_0+a)b$$

defines it very accurately,  $P_0$ ,  $a$  and  $b$  being constants characteristic of the active state of the muscle. This equation was established for maintained tetanic contractions. In single twitches the same kind of relation exists, the velocity of shortening being greater the less the load; but the rapid onset of relaxation obscures its simplicity, the full isometric tension, for example, not being nearly developed in a twitch, because relaxation sets in too early to allow the contractile component time to stretch the elastic one enough. The question naturally arises, how soon after the beginning of stimulation does the characteristic equation hold? Must we wait for the contraction to be fully developed? Or is it valid, with the same constants, right from the start? If it is, then there must be an abrupt change from rest to full

activity immediately after the latent period is over, not a gradual one as the ordinary myogram suggests. The purpose of the present investigation was to examine the mechanical condition of the contractile component at various moments after a maximal single shock; the method used was to apply a sudden stretch and measure the resulting rise of tension.

There is strong converging evidence that the change of state from rest to full activity occurs abruptly very early after a stimulus. Some can be found, and much of it has been referred to, in a recent series of papers (Hill 1949*a* to *e*) on the heat production in a muscle twitch. The chief and earliest evidence was the discovery by Gasser & Hill (1924), (*a*) that the initial development of tension in the tetanic contraction of a frog's sartorius is exactly similar in its time relations to the redevelopment of tension after a quick release at a later moment, by when the contraction certainly is fully developed, and (*b*) that a muscle suddenly becomes very inextensible to a quick stretch quite early after a shock, probably at the end of the latent period. The first point was later confirmed by Hill (1926*a*, *b*) in quick releases of the body muscle of *Holothuria* and of the biceps cruris of the tortoise; he found in tortoise muscle, like Gasser & Hill in frog's, that a sudden release of about 10 to 15 % of the muscle's length brought the tension to zero, from which it then redeveloped along a curve indistinguishable from that of the original development. The investigation was extended by the work of Levin & Wyman (1927). Further evidence is provided by the rapid initial outburst of heat which begins in a muscle twitch before any mechanical response is visible (Hill 1949*e*); the sudden onset of the 'heat of activation' led one to expect a very early and extensive change in the mechanical properties of the contractile component. This sudden mechanical change would not be evident in the ordinary myogram, since a force cannot be manifested externally until the contractile component has shortened enough to raise the tension in the series elastic component; and shortening is a slow process. It was necessary, therefore, to obviate the delay due to shortening, and this could be done by applying a rapid stretch at various moments after a shock. The object of the present experiments was to investigate the second finding of Gasser & Hill in greater detail, and in particular to determine whether the inextensibility reaches its full degree immediately at the end of the latent period, how long it lasts and how it declines in a single twitch.

#### METHOD

Simultaneous records were made, by a double-beam cathode-ray tube and a fixed camera, both of the stretch itself and of the change of tension resulting from it in the muscle. A revolving contact breaker opening three keys provided the timing. The first key operated two rapid relays arranged in series; one relay brightened the beams of the oscilloscope and started them sweeping, the other applied a condenser discharge to the muscle, the two events occurring at the same moment within a fraction of a millisecond. The second key operated the magnet which applied the stretch. The third key darkened the beams when the record was complete. The time between the opening of the first two keys could be adjusted to

within 1 msec., but the interval between the shock and the beginning of the stretch, and the duration of the stretch itself, were read directly on the record.

The armature of the magnet carried a stiff aluminium arm attached by a nylon thread to a light aluminium lever below it. A strong spring pulled the armature rapidly away from the magnet when the current through the latter was broken. The movement of the armature and its arm was damped by a vane hanging in a small bath of silicone fluid (DC200 supplied by Albright and Wilson). This liquid has a very high viscosity which is much less affected by temperature than that of most fluids; it is clean and simple to use. Damping is essential, to prevent vibration and chatter; it could be adjusted by raising or lowering the bath. The amplitude of the stretch was set by a screw stop arresting the lever. The lever was attached to a second aluminium lever below it by a 'strain gauge' (see below) consisting of a double length of  $50\mu$  double-silk-covered constantan wire. Both levers were carried on miniature ball-bearings. The lower lever was connected to the muscle by a thin straight wire, and carried a strip of aluminium foil which cast a shadow on two opposed rectifier photocells by which its movement was recorded. The whole equipment was fixed on an adjustable Palmer stand and could be raised or lowered by a screw to within 0.1 mm.

The speed of the stretch was adjusted by altering the spring and the damping; its duration could be varied as desired, from about 6 msec. upwards. Very rapid stretches involved more vibration and were found to be unnecessary.

The wire of the strain gauge was doubled back on itself (to avoid induced currents from the magnet) and lightly insulated with flexible bakelite varnish. The connexion, therefore, between the two levers consisted of two parallel  $50\mu$  wires, each 11 cm. long, mechanically in parallel but electrically in series. The equivalent mass of the lower lever was so small that its acceleration in a stretch caused little rise of tension, and such as occurred was evident only in the earliest stages of a stretch. The wire of the strain gauge (44 ohms) formed one arm of a resistance bridge. A tension in the wire changed its resistance proportionally and was recorded by the deflexion of a galvanometer of very short period (Hill 1948*a*). The sensitivity available was so high that considerable negative 'feed-back' to the galvanometer could be employed, and the time-lag in the tension record was not more than about 1 msec.

When Gasser & Hill (1924) applied quick stretches to a muscle they used a rapid lever of ordinary type to record the tension. The lever was light but its carriage was heavy and could not be moved suddenly, as the wire strain-gauge can be moved; it was necessary to apply the stretch to the other end of the muscle. The muscle, therefore, could not be immersed in Ringer's solution and kept in a thermostat without making the equipment much more elaborate. In the present experiments it was desired to work with muscles under very constant conditions, and particularly at 0° C, where their slower contraction makes the resolution in time of rapid events much easier. The use of the wire strain-gauge, with its negligible inertia, rendered it possible both to stretch the muscle and also to measure its tension, from one end only. The muscle, therefore, could be mounted in a suitable chamber and immersed in Ringer's solution in a thermostat.

The amplitude and duration of a stretch were recorded photoelectrically as described above. The output from the opposed photocells was amplified and recorded on the second beam of the cathode-ray tube.

The muscle (a sartorius from toad or frog) was held at its pelvic end in a clamp, and tied at its tibial end to a thin straight wire running up to the lever. It lay gently in contact with a block of Perspex in which twelve electrodes were embedded about 2 mm. apart. The electrodes were of silver and were flush with the face of the Perspex, the whole forming a smooth plane surface. Electrodes 1, 3, 5, 7, 9 and 11 were connected together, as were 2, 4, 6, 8, 10 and 12. One group formed the cathode, the other the anode, of the stimulus (a supermaximal condenser discharge). By this arrangement the muscle was stimulated simultaneously at a number of points throughout its length. At 0° C contraction is propagated only at about 40 cm./sec., and in a muscle 3 cm. long stimulated at a single point in the middle the ends would not be active (except in so far as nerve fibres also were excited) till 30 to 40 msec. later. It was desired to analyze events occurring in considerably shorter times than this, and the results would have been confused if a quick stretch had been applied to a muscle different parts of which were in different stages of contraction, or not yet active. The muscle, in Ringer's solution, was in gentle contact only with the multiple electrode surface, and no significant resistance can have occurred there. It was easy to test any possible faults of this kind by stretching an unexcited muscle; the effect of such stretches was always small.

The advantage of toads' muscles is that they contract at only about half the speed of frogs' muscles, so making easier the resolution in time of rapid events. Moreover, they shorten more and pull up better on stimulation to a shorter length. Most of the experiments, therefore, were made with toads' muscles at 0° C. Some experiments, however, were performed on frogs' muscles; and a few were made on both kinds of muscle at 10 to 15° C. Similar results were obtained in all of them.

The procedure was as follows. The muscle was set up in its chamber and all connexions made. It was then left in oxygenated Ringer's solution for several hours at 0° C, usually overnight, in order to get into a steady state. The length of the muscle at rest under a small load (usually about 1 g.) was noted, and the height of the stand was set so that stretches brought the muscle to a length not greater and usually less than this. The amplitude of the stretch was set by the screw stop under the upper lever, and if that was altered the stand could be raised or lowered so that the stretched length remained the same. The resting tension of the stretched muscle was always small. Before recording a contraction the current through the magnet was made and the muscle released to its shorter length under zero load. A few shocks were then applied to make it draw up as completely as possible to that length. The interval between shock and stretch was set on the arm opening the second key. A record was then made. For comparison with the 'stretched twitch' another record was made for which the muscle was similarly stretched shortly (usually 70 msec.) before the shock was applied. This record did not differ significantly from one in which the muscle was stretched a long time before being stimulated.

There are four possible variables. Of these the two most important are the amplitude of the stretch and the interval after the shock at which it is applied; the

other two are, the length to which the muscle is stretched and the time occupied in the stretch. The records of experiments given in figures 1 to 6 below show how these affect the result. The duration of the stretch is of consequence only in so far as the muscle can go on shortening (stretching its series elastic component) while the stretch proceeds. Fairly rapid stretches therefore, are desirable, but not so rapid that vibration is set up.

### RESULTS

In figure 1, *A* and *B*, are curves, copied directly from photographic records and superimposed, of the tension in a muscle stretched about 15 %, (*a*) 70 msec. before a single maximal shock and (*b*) just after the end of the latent period. *A* was for a frog's sartorius at 0° C, *B* for a toad's sartorius at 15° C. In *A*, for which the latent period was about 24 msec., the stretches (*b*) occurred between 35 and 50 msec. (measured from the shock); in *B*, for which the latent period was about 10 msec., between 12 and 25 msec. In both the tension rose abruptly to a peak, which was reached precisely at the moment when the stretch ended, then dropped back

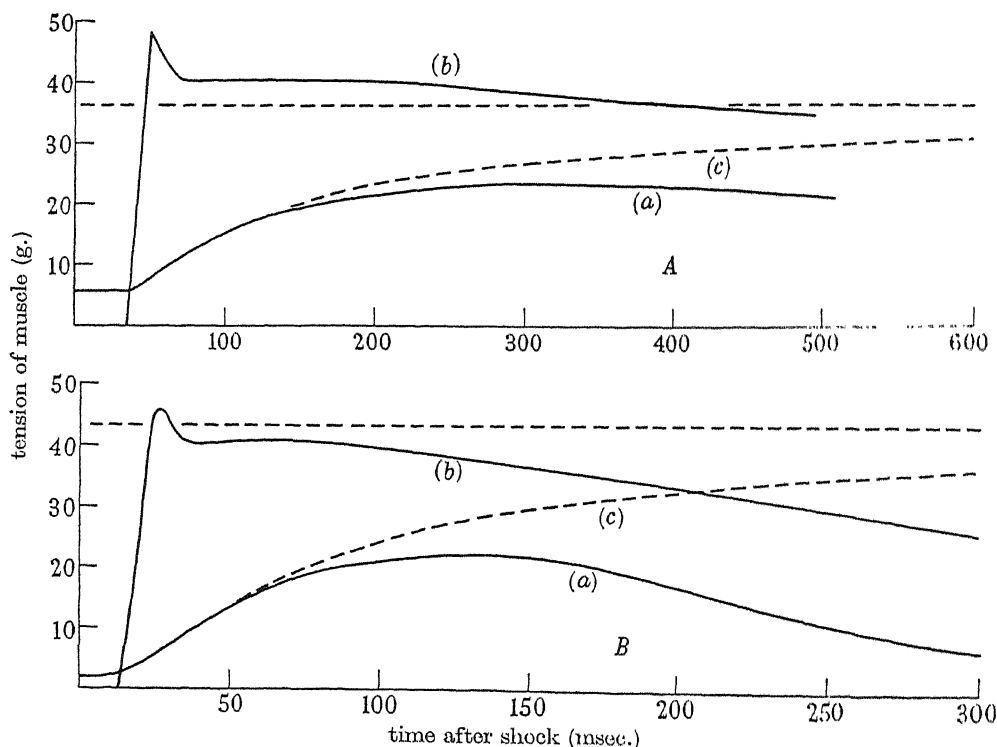


FIGURE 1. Effect of stretching a muscle about 15 %, shortly after stimulation 'all over'. *A*, frog's sartorius 0° C, 114 mg., 37 mm. under 2.6 g.; stretch 5.5 mm. from 31.2 to 36.7 mm.; latent period about 24 msec.; 50 g. tension equivalent to 1.5 kg./cm.<sup>2</sup>. *B*, toad's sartorius 15° C, 65 mg., 31 mm. under 1.7 g.; stretch 4.5 mm. from 25 to 29.5 mm.; latent period about 10 msec.; 50 g. tension equivalent to 2.4 kg./cm.<sup>2</sup>. Records (*b*), muscle stretched after maximal shock, during interval (*A*) 35 to 50 msec., (*B*) 12 to 25 msec. Records (*a*), for comparison, muscle stretched 70 msec. before maximal shock. Records (*c*), isometric tetanus at stretched length, broken line at top showing final level of tension reached.

quickly to a long plateau from which it fell gradually during relaxation. The tension at the plateau was about twice that in the twitch of a muscle stretched before stimulation—or not stretched at all—and about equal to that in a maximal isometric tetanus (curves (c)) applied at the stretched length.

It is necessary to dispose at once of the possible objection that the stretch acts as a 'stimulus', as it can do in smooth muscle (e.g. of *Holothuria*) (Hill 1926*b*). There are five reasons against this: (i) the sudden rise of tension produced by the stretch is altogether faster than that set up by a stimulus, (ii) the rise of tension increases with the amount of stretch up to a certain limit (beyond which the muscle 'gives' or 'slips') but is little affected, within limits, by the speed of stretch, (iii) the stretch of a muscle otherwise unstimulated produces only the small effect which can be observed on stretching any visco-elastic material, (iv) a muscle stretched just before stimulation gives practically the same response as if stretched a long time before, there is no summation, and (v) a stretch applied during relaxation, when a stimulus would give a summated contraction, produces only a small transient result. It will be assumed, therefore, that the effect of a stretch, applied to striated muscle, is purely mechanical. If a stimulus did result it would be apparent in the records, as it was in the body muscle of *Holothuria*.

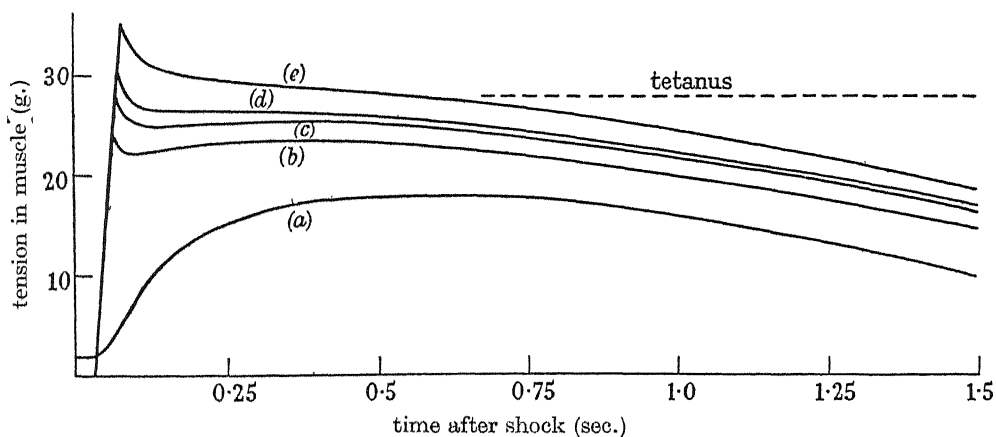


FIGURE 2. Effect of stretching a muscle various amounts at the same moment shortly after a maximal shock. Toad's sartorius  $0^{\circ}\text{C}$ , 62 mg., 36 mm. under 1.7 g.; latent period 32 msec.; 30 g. tension equivalent to  $1.8\text{ kg./cm.}^2$ . Stretch started in (b), (c), (d) and (e) 34 msec. after and in (a), for comparison, 70 msec. before, the shock. Stretches as follows: (a) 4.3 mm., (b) 3.9 mm., (c) 4.3 mm., (d) 4.7 mm., (e) 5.1 mm. Final stretched length 36 mm. throughout. Broken line, final level of tension in isometric tetanus at length 36 mm.

In the experiment of figure 2 a toad's sartorius at  $0^{\circ}\text{C}$  was stretched various amounts, starting always at the same moment just after the end of the latent period and finishing always at the same length. The shorter stretches, (b) and (c), were not enough to give the full effect, and the tension continued to rise slightly after the stretch. The longest stretch, (e), was rather too much and the tension fell slowly throughout. With 4.7 mm. stretch (about 13 %, curve (d)), after a rapid fall from the peak the tension remained on a plateau, then slowly relaxed. The level of (d)

was slightly less (about 6 %) than the full tension developed in a tetanus. (Curve (a), as before, is the record of an isometric twitch stretched 4.3 mm. 70 msec. before the shock.

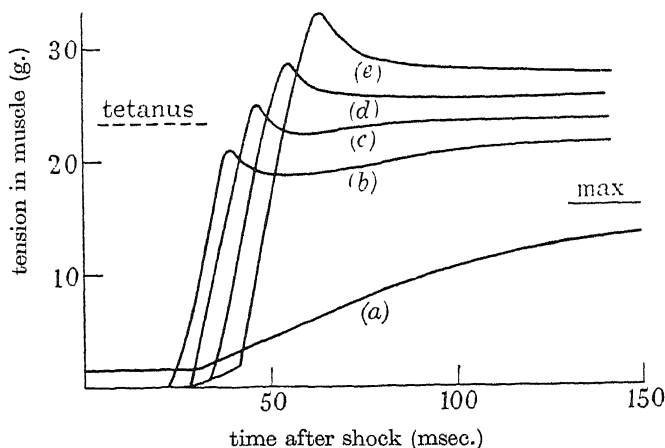


FIGURE 3. Effect of stretching a muscle the same amount at various times after a maximal shock. Frog's sartorius  $0^{\circ}\text{C}$ , 69 mg., 34 mm. under 2 g.; latent period 22 msec.; 30 g. tension equivalent to  $1.5\text{ kg./cm.}^2$ . Stretch 3.1 mm., from 28.6 to 31.7 mm.: (b) 22 to 38 msec. after shock, (c) 28 to 44 msec., (d) 33 to 51 msec., (e) 42 to 61 msec.; (a) for comparison, 70 msec. before shock. Broken line, final level in isometric tetanus at length 31.7 mm. 'max.' denotes maximum of (a).

In the experiment of figure 3 a frog's sartorius at  $0^{\circ}\text{C}$  was stretched the same amount at various times after a maximal shock. The amount of stretch was too small (10 %) to give the full tension if applied very early (curves (b) and (c)). If the stretch was applied later the contractile component of the muscle had time to shorten somewhat, stretching the elastic component before the external stretch occurred; the total stretch, therefore, was greater. In curve (d) the plateau was attained at a level about 9 % higher than that of an isometric tetanus. In curve (e) the total stretch, internal plus external, was too great, and the tension fell throughout. Figure 4 shows a similar experiment on a toad's sartorius. The stretches (about 12 %) were slower than in the other figures. Applied very early (curve (b)) and completed before the end of the latent period a stretch had little effect. Beginning before and finishing after the end of the latent period (curves (c) and (d)) a stretch had a greater effect. Later still, beginning after the end of the latent period (curves (e), (f), (g)), a stretch had a greater and greater effect. In curve (e) the stretch was very slightly too early to give the plateau, in curves (f) and (g) too late.

In the experiment of figure 5 the same stretch was applied at the same time, but from different initial lengths. A plateau would have been reached somewhere between (e) and (f). From a shorter initial length the effect of the stretch was less. The reason for this is probably that at shorter initial lengths some of the fibres of the muscle were slack, the first result of the stretch being merely to straighten them out; until they were straight the stretch would have no effect on their tension, and the remainder of the stretch would then be too small to give the full rise.



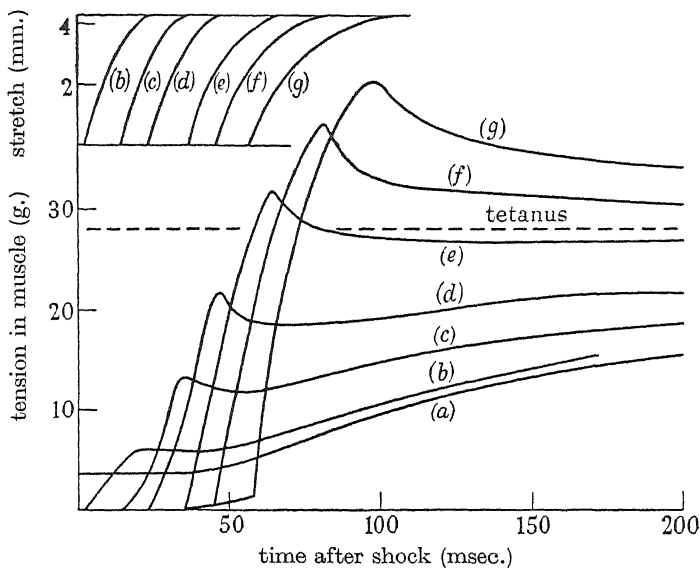


FIGURE 4. Effect of stretching a muscle the same amount at various times, after a maximal shock. Toad's sartorius  $0^{\circ}\text{C}$ , same as in figure 2. Stretch  $4.3\text{ mm.}$ , from  $31$  to  $35.3\text{ mm.}$ , at various times as shown in the small diagram above, starting at (a)  $-70\text{ msec.}$ , (b)  $2\text{ msec.}$ , (c)  $14\text{ msec.}$ , (d)  $23\text{ msec.}$ , (e)  $36\text{ msec.}$ , (f)  $45\text{ msec.}$ , (g)  $56\text{ msec.}$ . Broken line, final level in isometric tetanus at length  $35.3\text{ mm.}$  The stretches in this series were relatively slow.

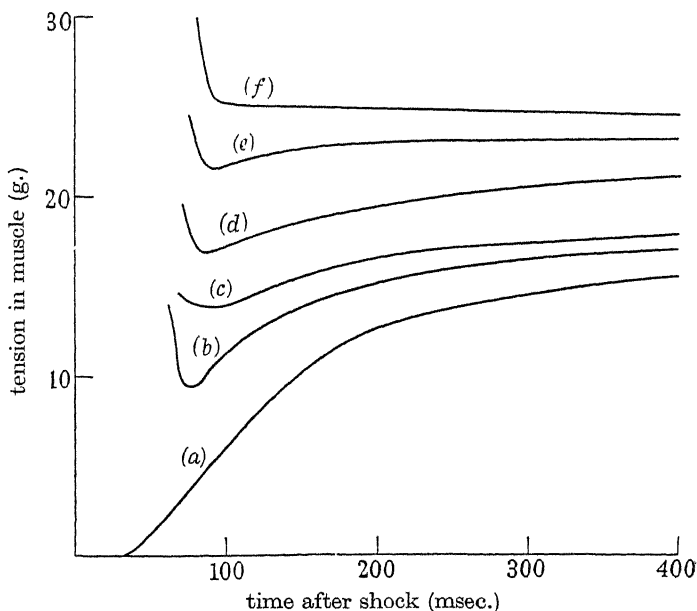


FIGURE 5. Effect of stretching a muscle the same amount between different lengths after a maximal shock. Toad's sartorius  $0^{\circ}\text{C}$ ,  $42\text{ mg.}$ ,  $31\text{ mm.}$  under  $0.9\text{ g.}$  load: latent period  $29\text{ msec.}$  Stretch  $3.9\text{ mm.}$  starting at  $55\text{ msec.}$  and ending at  $64\text{ msec.}$ , between lengths as follows: (b)  $22.1$  to  $26\text{ mm.}$ , (c)  $23.1$  to  $27\text{ mm.}$ , (d)  $24.1$  to  $28\text{ mm.}$ , (e)  $26.1$  to  $30\text{ mm.}$ , (f)  $27.1$  to  $31\text{ mm.}$ . Record (a) is of a twitch at  $30\text{ mm.}$  Note. The initial rising phase of curves (b) to (f) is omitted in order to avoid the confusion of five lines nearly but not quite coincident.

In the experiment of figure 6 the same stretch (9 %) was applied at various times throughout a twitch. On the rising phase, early stretches produced the result already described, later ones caused the muscle to 'give'. Applied during relaxation, a stretch produces little more than a transient effect, smaller the later the moment of application. Late stretches find the muscle in a plastic state, offering little resistance.

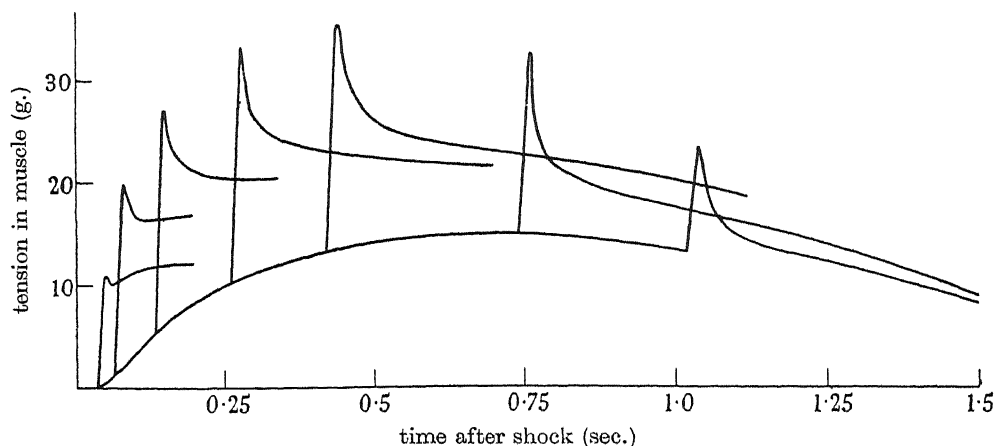


FIGURE 6. Effect of stretching a muscle the same amount at various times. Toad's sartorius  $0^{\circ}\text{C}$ , 45 mg., 33 mm. under 0.9 g. Latent period 30 msec. Stretch 3 mm. (30 to 33 mm.) starting at times 35, 64, 132, 260, 420, 740 and 1020 msec. and taking 15 to 20 msec. Later stretches have still smaller effects than the latest shown. 30 g. tension equivalent to  $2.3\text{ kg./cm.}^2$ .

The approximate equality of the maximum steady tension produced in a single twitch by a rapid stretch, to the full tension developed in an isometric tetanus, has been illustrated in figures 1 to 4. In six experiments at  $0^{\circ}\text{C}$  the average ratio of the former to the latter was 1.15; in five experiments from  $11$  to  $15^{\circ}\text{C}$  it was 0.88. The differences from unity may be real, or they may depend on the experimental difficulty of finding a stretch which is just large enough to obviate the necessity of any further shortening of the contractile elements, yet not so large as to cause the contractile component to 'slip'. It is not easy to hit the exact balance, particularly in a muscle made up of several hundred fibres with properties varying from one to the other, or probably between different regions of a single fibre. It has been assumed that the target to aim at is a tension curve running horizontal for a time after the stretch. It might be the case that the strength of the contractile component is greatest at the start, immediately after the abrupt change from rest to activity, and that 'relaxation' really begins at once. Curve (e), figure 2, for example, might be a more appropriate index of the changing internal state of the muscle than curve (d) or (c); or than curve (b), figure 1*B*. It is impossible, therefore, on the present evidence, to insist that stretching a muscle appropriately, during a single twitch, gives precisely the same tension as is developed without external stretch during a prolonged contraction at the same length. It is certain, however, that the

two are nearly equal, and in the absence of further evidence their equality will be assumed.

The reason why the tension in a normal isometric twitch is so much less than in a tetanus is easy to see. The nature of the relation between speed and load embodied in the characteristic equation is that, with greater loads verging on the final isometric value, the speed of shortening is very low (see figure 8). The rise of tension, therefore, in the later stages of an isometric tetanus is very protracted. Consequently in a twitch, owing to the early onset of relaxation, there is too little time for the full amount of shortening to occur; without it, the elastic component cannot be stretched enough to give the full tension potentially available at the start in the contractile component.

#### *The latent period*

It has been shown that a muscle suddenly becomes much less extensible very soon after the end of the latent period. The latent period was measured in an isotonic contraction with small load, otherwise under the same conditions as obtained in the experiments described above, in particular with a supermaximal stimulus applied at 12 electrodes distributed along its length. Very little time, therefore, was wasted in the propagation of contraction. Records were made, as before, of the movement of the shadow of the lever on a pair of opposed photocells. Magnification was high. 20 to 30 times, and photographs could be read to 0.1 mm.; shortening was detected, therefore, when it reached 3 to 5  $\mu$ , one or two parts in 10,000 of the length of the muscle. The speed of recording was 1 to 1.5 mm./msec. The inertia of the lever, etc., was negligible at the speeds involved.

It could not be expected, however abrupt the change from rest to activity might be in the individual muscle fibre at the point stimulated, that the trace recording the contraction should leave the base-line suddenly at an angle. The latent period is certain to vary somewhat from one fibre to another, probably also from one region to another in the same fibre, and this is bound to produce a rounding of the 'take off' from the base-line and make the latent period slightly indefinite. The measurement, however, of a number of records, and the close agreement found, made it clear that such uncertainty as exists is relatively small. In five toad *sartorii* at 0° C the observed values of the latent period varied from 29 to 32 msec., with a mean of 30 msec. The upstroke, starting tangentially from the base-line and then bending upwards, became linear at about 45 msec. We may suppose either (a) that it took up to 15 msec. for the state of activity to be fully developed, or (b) that the latent period of individual fibres, or of different regions of them, varied up to this amount from its shortest value. Probably both causes contributed to the initial rounding of the upstroke. The fact, however, that the velocity of shortening of the whole muscle was almost fully developed within 1½ times the shortest latent period that could be observed strongly confirms the conclusion from the quick stretches that the state of full activity is reached soon after the latent period is over. In frog's muscle at 0° C the latent period was about 24 msec., and the records of shortening became linear at about 36 msec., times rather shorter than in the toad but leading to the same conclusion. These latent periods are considerably greater than those recorded at 0° C by the piezo-electric method at the actual site of

stimulation (Abbott & Ritchie 1948). What the difference is due to is not clear. It was necessary, however, to measure the latent period under the same conditions as obtained in the other observations with which it was to be compared.

The earliest moment, at 0° C, at which the heat production can be detected is, in the frog's sartorius about 10 msec., in the toad's about 25 msec., after a shock (Hill 1949*e*). In both, the heat production, which starts at its maximum rate, is well under way before the mechanical response appears. The early outburst is 'heat of activation' (Hill 1949*a*), the 'heat of shortening' coming later. Presumably, the heat of activation is a by-product of the reactions by which the sudden change of state from rest to activity is effected.

### DISCUSSION

It is very noticeable, in figures 1 and 2, that the tension existing in a stimulated muscle after being stretched is greater than that of a normal twitch, not only initially but throughout relaxation. If the tension to which a muscle is stretched is just equal to the maximum it can bear, then as the intensity of its active state declines that tension becomes too great and the contractile portion begins to be stretched by the elastic portion. Now it is known that when a muscle is subjected to a load rather greater than it can bear, it slowly lengthens (Levin & Wyman 1927; Katz 1939). According to Katz the speed of lengthening is several times less than could be expected from its rate of shortening under a rather smaller load; a discontinuity occurs at zero speed in the characteristic relation between force and velocity (see, for example, his figure 5). Expressed in another way, the force required to make a muscle lengthen is greater than the full isometric tension by an amount several times larger than we should expect from the characteristic relation extrapolated back through the horizontal axis (see figure 8, below). The effect can be seen in the paper by Levin & Wyman, particularly in their figure 2 containing records, made on a dog-fish muscle, of the force exerted at various constant speeds of shortening or lengthening.

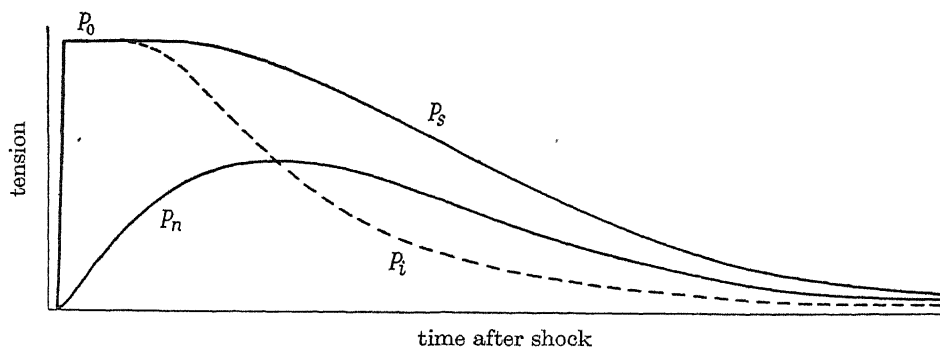


FIGURE 7. Diagram of mechanical changes during a muscle twitch.  $P_n$ , tension in normal twitch.  $P_i$ , intrinsic strength of the contractile component at any moment, i.e. the tension at which it neither shortens nor lengthens.  $P_s$ , tension in muscle stretched quickly immediately after the latent period by such an amount that its contractile component at first neither lengthens nor shortens.  $P_0$ , initial value of  $P_i$  and  $P_s$  (cf. Gasser & Hill 1924, figure 16.)

Now it is natural to regard a relaxing muscle as having the same kind of properties as a fully active one, but of diminishing intensity. During isometric relaxation the elastic component gradually pulls the contractile component out again until they both reach their original length. Let  $P_i$  (figure 7) be the force which the contractile component could just bear at any moment without shortening or lengthening. Its initial value is  $P_0$ , the full isometric tension in a maintained contraction. As the tension  $P_n$  rises in the early stages of a normal twitch, the contractile component stretching the elastic one,  $P_n$  and  $P_i$  gradually approach one another, one rising and the other falling until they become equal at the moment when the tension of the muscle reaches its maximum. Beyond the maximum the process is reversed and the elastic component stretches the contractile one, the latter resisting extension with a force greater than  $P_i$ . On one side of the maximum  $P_n$  is less than  $P_i$  because the contractile component is shortening, on the other side  $P_n$  is greater than  $P_i$  because it is being extended.

Now consider what happens when the muscle is suddenly stretched to such a length that the full tension  $P_0$  is just reached. As soon as relaxation starts the strength  $P_i$  of the contractile component becomes less than  $P_0$ , so it begins to be stretched. It resists with a force  $P_s$  greater than  $P_i$ . As relaxation proceeds  $P_s$  falls, always lagging behind  $P_i$  and remaining above  $P_n$ . The curves of  $P_s$  and  $P_n$  do not cross, they can only approach one another gradually as relaxation goes on.

In an isotonic contraction the elastic component remains at constant length, and shortening occurs according to the characteristic relation between force and speed. The maximum shortening is reached when the intensity  $P_i$  of the active state has fallen to a value equal to the load. With a small load the shortening may proceed so far that  $P_0$ , the initial value of  $P_i$  at the shorter length, itself becomes less, and this helps to diminish  $P_i$  as well as its decline with time. With large loads and small shortenings  $P_0$  remains nearly constant, and the fall of  $P_i$  is due simply to relaxation. It is well known that with large isotonic loads relaxation occurs very suddenly. The maximum shortening is reached at a time when  $P_i$  is falling rapidly, and as soon as  $P_i$  becomes less than the load the contractile component begins to lengthen. This, however, does not relieve the tension, as it does in an isometric contraction, and as  $P_i$  continues to fall the load becomes relatively so great that the contractile mechanism 'gives' and a very rapid extension results. It is under these conditions that the relaxation heat, derived from the energy of the load, is most sudden and obvious (Hill 1949*b*).

A more exact description of the process cannot be given at present, owing to lack of quantitative knowledge of the changing mechanical properties of the contractile component during relaxation. It is clear, however, from the curves of figures 1 and 2 that the intensity  $P_i$  of the active state has begun to decline well before the moment at which the tension is a maximum in a normal isometric twitch; and it is obvious on general grounds that  $P_i$  and  $P_n$  must become equal at that moment, as, indeed, was indicated in figure 16 of the paper by Gasser & Hill (1924). Taking account of all the evidence, the true relations cannot be very different from those sketched in figure 7.

*The series elastic component*

If the stimulus is not repeated the active condition of a muscle gradually declines, as illustrated in figure 7, its characteristic properties passing back continuously in 'relaxation' to those of the resting plastic state. If the stimulus is repeated at an appropriate low frequency the active state can be fully maintained for a considerable time, in a toad's muscle at 0° C for minutes. Each shock restores the value of  $P_i$  to its full height.

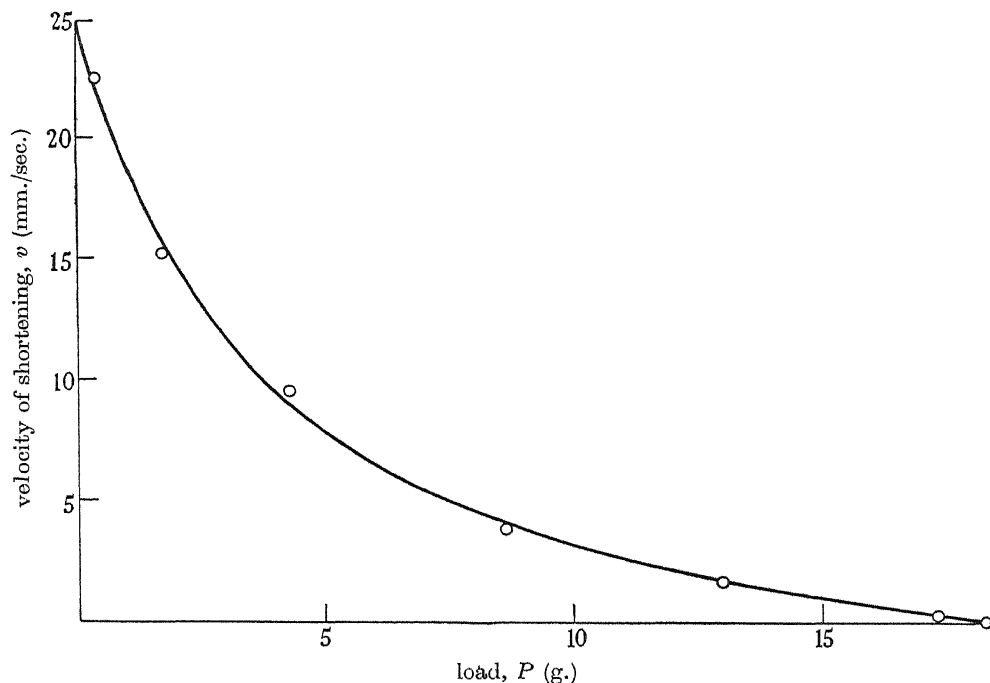


FIGURE 8. Relation between speed of shortening and load, in contraction of toad's sartorius at 0° C. Muscle 40 mg., 29 mm. long under 0.9 g. Tetanus at initial length 26 mm. under various loads, or isometric. Circles, observed points: curve calculated from equation  $(P + 3.8)(v + 5.2) = 11.5$  ( $P_0 = 18.3$ ,  $P_0/a = 4.8$ ,  $b = 0.2$  length/sec.).

Given the form of the characteristic relation between force and speed of shortening, the stress-strain relation of the series elastic component can be calculated from the myogram of an isometric tetanus. We assume that the initial tension is small, so that the effect of the parallel elastic component is negligible. Let  $y_e$  be the total extension of the series elastic component up to any moment after the start; then the speed of shortening of the contractile component at that moment is  $v = dy_e/dt$ . Let us determine experimentally the characteristic relation between force and speed and plot it in a curve like that of figure 8. Now  $P$  is known at every moment during an isometric tetanus, so that, from the characteristic relation, the corresponding value of  $dy_e/dt$  can be read off. Plotting  $dy_e/dt$  against  $t$ , it is simple, by measuring areas and assuming  $y_e = 0$  at  $t = 0$ , to calculate  $y_e$  at all times during contraction. But  $P$  also is known at all times, so that the relation between  $y_e$  and  $P$  is determined.

It will be noted that no assumption has been made as to the exact form of the characteristic relation, only that it can be determined experimentally. If the characteristic equation is obeyed the calculation can be made as follows. We write  $dy_e/dt = b(P_0 - P)/(P + a)$ ; then,  $P$  being known from the isometric myogram as a function of time,  $dy_e/dt$  can be calculated and we proceed as before.

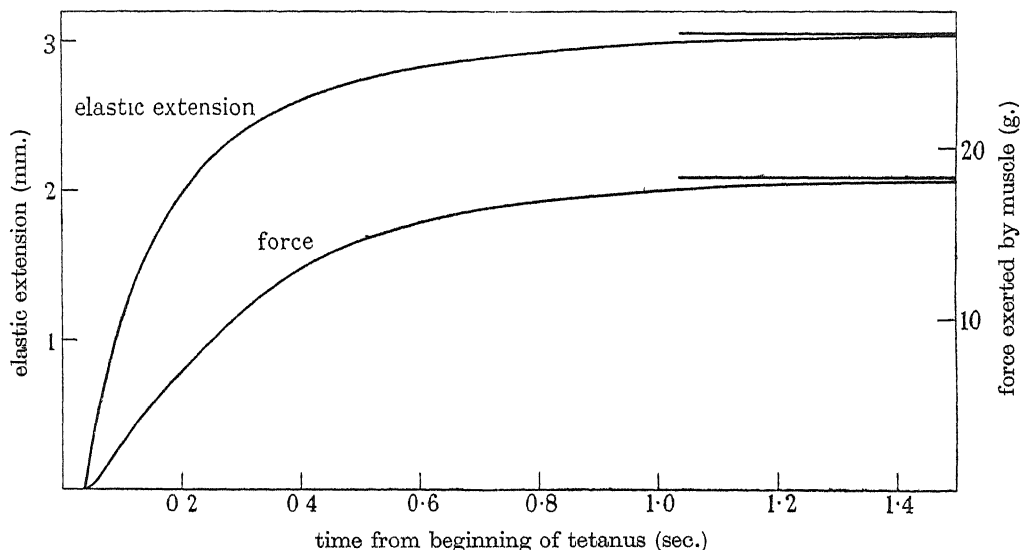


FIGURE 9. Lower curve, isometric contraction of toad's muscle referred to in figure 8. Upper curve, total extension of series elastic component calculated as described in text. The final levels of both curves are shown on the right.

Figures 8, 9 and 10 show how this works. In the isotonic tetanus of a toad's sartorius at  $0^{\circ}\text{C}$  the velocity of shortening was measured for various after-loads; the initial tension was very small. The observations are shown by circles in figure 8, together with a curve calculated from the equation; the agreement is good. In figure 9 the lower curve represents an isometric contraction recorded under identical initial conditions; the upper curve gives the relation, calculated as above, between  $y_e$  and  $t$ . From figure 9 simultaneous values of  $P$  and  $y_e$  were read off and plotted against one another in figure 10, giving the stress-strain curve of the series elastic component. Its general nature is as expected, a high extensibility at small loads, a much lower one at greater loads, the relation becoming linear in the upper range. Wilkie (1949) has used a similar method for calculating, from the isometric tension curve of human arm muscles exerting a maximal effort, the compliance of their series elastic component at various loads. He also has found that the compliance is much greater at small loads than at high ones.

The load-extension curve of figure 10 cannot be obtained by stretching a muscle at rest, the tension then being due chiefly to the parallel elastic component. It is possible, however, to get the relation of figure 10 in another way during a maintained contraction, by applying quick releases and recording the sudden drop of tension. If we measure the tension after release, from the records of figure 4 for

frog muscle in the paper by Gasser & Hill (1924), or of figure 3 for tortoise muscle in the Croonian Lecture by Hill (1926*a*), and plot it backwards against the distance released, we obtain a relation exactly similar to that of figure 10. As stated by Gasser & Hill, the tension drops to zero if the muscle be suddenly released by as much as 10 to 15% of its length. The maximum extension in figure 9 is 10 %.

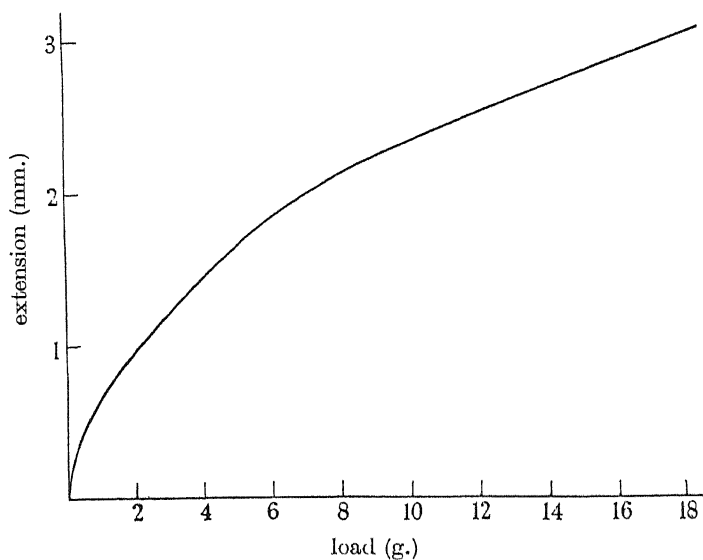


FIGURE 10. Stress-strain curve of series elastic component of muscle referred to in figures 8 and 9; calculated as described in text.

Knowing the force-extension curve of the series elastic component and the force-velocity relation of the contractile component it is obviously possible to reverse the procedure and calculate the form of the myogram under any desired external conditions, for example with a load varying with time. With large amounts of shortening the calculation would be complicated by the fact that the speed of shortening would be a function not only of the load but of the length. The necessary information could be derived from a single series of isotonic contractions under various loads, and exhibited as a family of curves each like that of figure 8 but for different lengths. It is scarcely necessary, however, to carry out such calculations; the fundamental mechanical properties of muscle are the important matter and they are already apparent.

The absolute length of the series elastic component is not known, nor has any method of measuring it been proposed. According to Ramsey & Street (1940) the isolated fibre of a frog's semi-tendinosus can shorten to 20 % of its length, so leaving very little room for anything but the contractile part; therefore, we might argue, the series elastic component must lie in the tendon and tendon bundles alone. This, however, disregards the possibility that the series elasticity in question may really be a property of the contractile component itself. Like any other body capable of bearing a stress, the contractile structure, during activity, might naturally be expected to show a corresponding strain, to possess an elasticity in its



own right. The series elasticity, therefore, though it must certainly reside partly in tendons and tendon bundles, may be due in part to the elasticity of the active contractile structure itself. If so, the series elastic element might reasonably be regarded as inherent in the whole muscle and the question of its absolute length would not arise. All we can really determine is the load-extension curve, and several independent lines of experiment converge to show that in the striated muscle of frog or toad the total extension under the full isometric tension is of the order of 10 to 15 % of the length of the muscle as a whole. This applies to muscles with parallel fibres running approximately from end to end; in muscles like the gastrocnemius the situation, for anatomical reasons, is far more complex.

Levin & Wyman (1927), using a variety of muscles (dog-fish, *Holothuria*, *Echinus*, spider crab and tortoise), examined the effect of speed of shortening or lengthening on the force exerted and the work done in a maintained contraction. The results were compared with those given by a model consisting of a damped spring in series with an undamped spring, the former representing the contractile component, the latter the series elastic one of the present discussion. The general agreement was good, and the model reproduced the phenomenon observed in muscle that, after release, the initial slope of the line relating force to distance is independent of speed and temperature; this itself is convincing evidence, as Levin & Wyman insisted, of the presence of an undamped series elastic component. We know now that a damped spring cannot properly represent the contractile component of muscle; in the former the relation between load and speed is linear, in the latter it follows the characteristic equation which is significantly different (figure 8) from a straight line. There are other reasons, fully discussed elsewhere (Hill 1938, 1949*a* to *e*), for discarding the simple visco-elastic model of the active contractile component of muscle. Replacing it, however, by the current dynamic concept of the active state, with its characteristic relation between load and speed, the substance of the argument of Levin & Wyman's admirable paper still stands. It is notable that they explained the form of the isometric tension-time curve by assuming 'that the viscous-elastic elements are the actual contractile structures and that they change their elastic properties suddenly on stimulation and start thereon to shorten and to stretch the elastic elements until a new equilibrium is reached'.

#### *'Slip' under excessive tension*

When an active muscle is stretched so much that its tension rises to a value greater than it can bear it 'slips' or 'gives'. The phenomenon has been referred to in various papers (Gasser & Hill 1924; Levin & Wyman 1927; Hill 1938; Katz 1939). All the curves of tension in figures 1 to 6 above rise initially to a peak which came exactly at the moment when the stretch ended. The effect was transitory and the tension fell back rapidly to a smooth curve characteristic of the changing internal state of the contractile component. If a muscle consisted only of a single fibre of uniform contractile strength throughout, it should be possible to stretch it by such an amount that the tension rose to its final value without overshoot. With several hundred fibres of varying length, cross-sectional area and strength, and without any certainty even that strength and area are the same at different points of a single

fibre, we should expect to find that a stretch which brought some fibres, or parts of a single fibre, to their maximum tension would be too great for other fibres or parts. That is presumably the reason why, with any but very small stretches, the overshoot occurs.

The development of the active state, in spite of its apparent abruptness, must in reality take a certain time. If a stretch were in progress during the transition the muscle would 'slip' at a lower tension than when the active state was fully developed. If, on the other hand, a stretch is applied (figure 6) late in a twitch it finds the elastic component already extended and the active strength of the contractile component waning; consequently, here the overshoot of tension is most evident.

The decline of tension after overshoot is attributed to 'slip', such as occurs in a wire, or in a thread of long-chain polymer such as nylon or polythene, strained beyond its elastic limit. The 'slip' is analogous to 'cold drawing', which in nylon or polythene leads to an orientation of the crystals parallel to the axis of the thread. In 'cold drawing' a considerable amount of heat is produced in the material, derived mainly from the mechanical work done in the irreversible stretch. The same is true of muscle. The heat production of frog and toad muscles has been measured under conditions analogous to those of figure 6. A stretch of 5 to 10 % of the muscle length applied early causes little overshoot and little extra heat production. Up to about the time when the tension, before stretching, reaches its maximum, overshoot and heat are both greater the later the moment of stretch. In a stretch applied after the maximum, the overshoot and the heat are each less, both being quite small when relaxation is well advanced. The heat appears mainly after a rapid stretch, during the time when the tension energy of the overshoot is being dissipated.

#### *The heat of shortening*

In a normal isometric twitch the contractile component shortens against the gradually rising tension of the series elastic component. If a muscle is suddenly stretched early in a twitch this shortening is slowed or prevented, according to the amplitude of the stretch. The heat of shortening, referred to elsewhere (Hill 1949*a*), makes up a substantial fraction of the total heat, and should be diminished or abolished by an early stretch. It was a crucial test to see if this was so. In figure 11 are records of the heat production (for details of method see Hill, 1949*d*) up to 0.6 sec. in three maximal twitches of a pair of toad sartorii at 0° C. In all three the final length was the same and about equal to the length in the body; the resting tension at that length was very small. *A* is the heat record of an isometric twitch at the greater length. For *B*, the muscle was first released 2.4 mm. and stimulated several times at the shorter length to cause it to draw up. The record was then made with the muscle stretched 2.4 mm. in the interval 58 to 70 msec. after the shock. The tension, not recorded, must have followed a course intermediate between the first and second stretches of figure 6. The overshoot of tension cannot have been large, and the extra heat shown by the rise of *B* above *A*, during and shortly after the stretch, was small. After that was complete (probably at about 0.09 sec.) curve *B* rose considerably more slowly than curve *A*, at a time when the tension in a normal twitch (see figures 2 and 6) was rising rapidly and the contractile com-

ponent shortening. After about 0.3 sec. the difference of slope disappeared, by then most of the shortening would have occurred. After that, curve *B* rose faster than curve *A*. The stretched muscle had a higher tension than the unstretched, and its relaxation heat was earlier and greater.

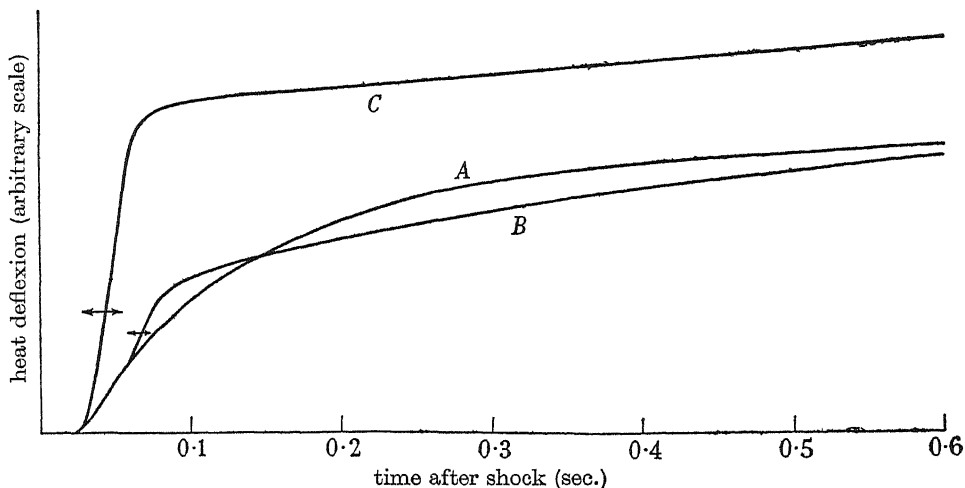


FIGURE 11. Heat production of a pair of toad's sartorii (63 mg.) at 0° C in a single maximal twitch. Curve *A*, isometric at length 26.5 mm. Curve *B*, initially at length 24.1 mm., stretched 2.4 mm. to length 26.5 mm. in the interval 58 to 70 msec. (shown by double arrows) after a shock. Curve *C*, initially at length 22.2 mm., stretched 4.3 mm. to length 26.5 mm. in the interval 28 to 52 msec. after a shock. Length of muscles in animal 26 mm., at rest under 1.3 g. 28.5 mm.

For *C*, the muscle was first released 4.3 mm. and stimulated as before. The record was then made with the muscle stretched 4.3 mm. in the interval 28 to 52 msec. after the shock. The stretch was considerable (19 % of the initial length) and started at about the end of the mechanical latent period; there must have been substantial overshoot of tension. The heat record rose very rapidly during and immediately after the stretch, but then settled down to a slope much less than that of *A*. Shortening must have been largely, if not wholly, prevented and shortening heat was absent. Later on, as with *B*, *C* rose more rapidly than *A* owing to the dissipation of the much greater tension energy of the stretched muscle. It was shown in an earlier paper (Hill 1949*a*) that the shortening heat can be largely abolished in an isotonic contraction by working with an extremely small load and causing the muscle to shorten up as much as possible by previous stimulation; very little more shortening can then occur, and the shortening heat is small. The converse experiment of preventing shortening by a sudden stretch leads to the same result. These experiments confirm the view that the heat described as 'heat of shortening' is, in fact, associated with shortening as such.

#### *The 'supernormal phase'*

The present results have a bearing on the observations of Hartree & Hill (1921) on the so-called 'supernormal phase' of muscular contraction. They applied two

maximal direct shocks in succession to a muscle under isometric conditions, and increased the interval between them from zero up to a value at which the two responses were separate. The response to the second shock was obtained by subtracting the ordinates of the response due to a single shock alone from those of the double response. The response to the second shock, estimated in this way, was initially zero (when the second shock fell within the absolute refractory period of the first one), increased gradually as the interval became greater, reached a maximum which was always well above the response to a single shock, and then gradually declined to the level of the latter.

We have seen above that shortly after a single shock a muscle changes its state abruptly from one of rest to one of full activity, in which the load it can bear is as great as the tension in a maintained tetanus. The intensity of the active state then gradually declines. A second shock can do no more than bring the active state back to its full intensity again. If this occurs at a moment when the series elastic component has already been considerably stretched the second response starts with an advantage which shows itself in the greater tension finally developed. The effect, indeed, is similar to that of a sudden stretch applied in a single twitch. If the strength of the active contractile component is raised to the same full value by the second shock as by the first one, it can make no difference whether a stretch is applied artificially from without or internally by a previous twitch. In either case the contractile component is relieved of the necessity of shortening so far before it can exert a given force, and thus has time to develop a greater one.

If this explanation is correct we should expect to find that a second shock leads to the greatest total tension if applied at the moment when the force developed in the first response is a maximum. Figure 7 in the paper by Hartree & Hill shows that this is the case: curves DII and EII in which the second response begins near the maximum of the first are higher than the rest. It is true that the difference, calculated as described above and shown by broken lines in Hartree & Hill's figure, is greatest when the second response begins rather later than the maximum of the first one. If, however, the above interpretation is correct, their method of calculating the second response as a difference is invalid. If the second shock abruptly restores the internal activity of the muscle to its full height, we cannot regard the second response as being displayed on the falling 'base-line' of the first one. According to the present view a completely new response is set up by the second shock, the greater tension then developed being due to the fact that the second response starts with the series elastic component already considerably stretched. The observations of Cooper & Eccles (1930) on the effect of two shocks applied at various intervals to the nerves of cats' muscles (gastrocnemius, soleus, internal rectus) appear to show that the total tension developed is greatest when the interval is least. Hartree & Hill stimulated their muscles directly. With indirect stimulation the risk is necessarily present that summation may occur at the neuro-muscular junction, and that the second impulse may activate muscle fibres which were not excited at all by the first one. Such summation would be greater with a shorter interval, and so might mask the purely muscular effect. It is impossible to eliminate this risk except by using supermaximal direct stimulation.

*The propagation of activity into the interior of a muscle fibre*

In a recent paper (Hill 1948) the question was discussed of how the state of activity set up by excitation, assumed to occur at the surface (for which there is very strong evidence), is propagated inwards throughout the cross-section of a muscle fibre. Is the active state at any point induced by the arrival there of some actual substance, e.g. calcium ions or acetylcholine, diffusing inwards from the surface where it was liberated by the stimulus? Or is it some physico-chemical event, rather than a substance, which travels? Diffusion is a relatively slow process and the time calculated for a diffusing substance to reach the interior from the surface is rather long. If we were able to assume (i) that only a fraction of the cross-section of a fibre is activated in a single twitch and (ii) that a considerable part of the contraction time is available for such activation, then diffusion might be fast enough to account for the observed speed with which muscles contract. If, however, the whole cross-section is activated in a twitch, diffusion would be too slow.

The present results allow a perfectly clear decision to be reached. The intensity of the active state in a twitch, as measured by the force which a muscle can bear after a quick stretch, is just as high as in a tetanus; it is impossible, therefore, to assume that only a fraction of the cross-section of each fibre is involved in it. Moreover, that active state is set up very abruptly, certainly within one-tenth of the time taken in a normal twitch for the maximum tension to be reached. These two factors together completely rule out diffusion as the effective agent. In a frog's muscle at 0° C, for example, the active state is fully developed within 40 msec. after a shock. With the symbols and quantities used in the previous paper, in a fibre of 100  $\mu$  diameter  $kt/a^2$  is 0.008 at 40 msec. and the relative concentration  $y/y_\infty$  in the middle, of any substance diffusing from the surface, would be zero to five places of decimals. Even half-way to the axis, involving only three-quarters of the cross-section, the value of  $y/y_\infty$  would be 0.003. At higher temperatures diffusion is rather more rapid, but contraction is very much quicker, and the calculation is even more decisive.

It is quite impossible, therefore, to explain the rapid development of full activity in a twitch by assuming that it is set up by the arrival at any point of some substance diffusing from the surface: diffusion is far too slow. Either we must suppose that excitation, in spite of converging evidence to the contrary, is produced by excitation (natural or artificial) throughout the interior, not merely at the surface: or we must look for some physical or physico-chemical process which is released by excitation at the surface and then propagated inwards. The latter seems much more likely. The speed of crystallization in a supercooled liquid 'seeded' at a point shows how rapidly such processes can travel.

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## Is relaxation an active process?

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Measurements of muscle heat production had indicated that relaxation is not an active process. Experiments to test this conclusion were made in two ways: (*a*) by measuring the mechanical latent period in isometric contractions over a wide range of lengths down to less than half the natural length in the body, and (*b*) by determining the relation between resting tension and length down to lengths at which the muscle became slack.

In a muscle under its resting tension alone the latent period after a shock remains nearly constant over a wide range of lengths. This range is extended by previous stimulation. If active relaxation occurred the latent period would be greatly increased.

A resting muscle exerts measurable tension down to 60 to 75 % of its natural length in the body. By previous stimulation at a shorter length the range of lengths within which measurable tension is exerted is increased.

In a muscle under zero external load lengthening occurs after contraction only from very short lengths. It is attributed to an elastic restoring force set up by the lateral expansion of the fibres.

The 'δ-state' described by Ramsey & Street in isolated fibres allowed to shorten too much is discussed. It may be due to mechanical damage to internal structures normally reinforcing the sarcolemma against expansion.

The nature of the contractile linkages in muscle is considered.

## INTRODUCTION

It is commonly believed that relaxation after contraction is an active process (see e.g. Heilbrunn 1943, p. 362), that a muscle, having shortened, forcibly lengthens again. The proof (Hill 1949*b*, and earlier papers) that no heat is produced in relaxation by a muscle relaxing without load or tension, and that any heat found otherwise during relaxation can be attributed to degraded mechanical work, threw doubt on this belief. Moreover, the heat of shortening (Hill 1949*a*) is very small in

a muscle excited at a very short length to which it has been brought by previous stimulation under a small load. It was possible that visible lengthening after contraction under such conditions was hindered, in an unloaded muscle on a thermopile, by capillarity, viscosity or friction. If so, however, we should expect an active relaxation to cause the fibres, or the fibrils, or possibly even the contractile molecular filaments, to push out into folds, which would have to be pulled straight when the muscle was next stimulated. Such taking up of slack could scarcely differ in character from ordinary shortening. The smallness, however, of the heat showed that little shortening occurred.

The latent period of muscle after a shock is an extremely sensitive indicator of 'slack' between the fixed support at one end of a muscle and a tension-recording device at the other. The latent period is brief, while the maximum speed of shortening, even in a muscle completely unloaded, is relatively low. If as little as 1 mm. of slack in a 25 mm. muscle had to be pulled out before a tension was manifested externally, the latent period would be multiplied several times. For example, in a toad's sartorius at 0° C the latent period is about 25 msec., and the maximum speed of shortening of the fully extended muscle is about equal to its length per second. A muscle, therefore, 25 mm. long would require about 40 msec. to pull out 1 mm. of slack, thus raising its apparent latent period to 65 msec. From a reduced initial length the maximum speed of shortening would be less, say 10 to 15 mm./sec., and the pulling out of 1 mm. of slack would lengthen the apparent latent period to 95 to 125 msec. When, therefore, a muscle had shortened under its resting elasticity, or as the result of previous stimulation, the presence or absence of slack could be established with considerable certainty by measuring the latent period.

In the first group of experiments described below the latent period was determined, over a range of lengths as wide as possible, on the short side of the resting length in the body. It was necessary, for accuracy, to avoid any influence of inertia, viscosity, stickiness, or capillarity; the muscle, therefore, was freely suspended in moist air, with an inextensible vertical connexion to a rapid, frictionless isometric tension-recorder. It had to be allowed to shorten freely to any desired length, without any force, however small, which could pull it out again. Experiments showed that over a considerable range of lengths the latent period is practically constant; and that this range can be increased by previous stimulation, causing the muscle to draw up without subsequent lengthening. If active lengthening occurred during relaxation, its effect on the latent period of the next response would be very much greater than anything observed.

A resting isolated muscle exerts a measurable tension over a considerable range of lengths less than its natural length in the body. In the second group of experiments the resting tension was measured, by a very sensitive method, over the whole range of lengths to which a resting muscle would shorten freely before it became slack. The resting tension is nearly enough an exponential function of length, diminishing to one-third for a 10 % reduction of length. At shorter lengths, therefore, the tension is very small and observed by ordinary methods might appear to be zero. In fact, however, a resting muscle continues to exert a measurable force down to about 60 to 75 % of its natural length in the body—which is less than its shortest possible

length in the living animal. The lower limit can be reduced still more by previous stimulation. Under this resting force it shortens up, if unloaded and unhindered, without significant lengthening of its latent period. By stimulation it can be made to draw together so that, pulled out to a greater length, it exerts considerably more tension at rest than it would otherwise have done at that length. The process can be repeated indefinitely. Let  $a$ ,  $b$  and  $c$  be three lengths,  $a > b > c$ . A muscle stretched to  $a$  and released to  $b$  exerts a force  $B_1$ ; released to  $c$ , stimulated at  $c$  and then stretched to  $b$  it exerts a force  $B_2$  considerably greater than  $B_1$ .  $B_1$  rises very slowly after release from  $a$  to  $b$ ,  $B_2$  falls gradually after stretch from  $c$  to  $b$ ; but  $B_2$  remains greater than  $B_1$  indefinitely, or until the muscle is stretched or stimulated. The fact that a muscle stimulated at a very short length, at which its resting tension is immeasurably small, can maintain a measurable resting tension afterwards at a slightly greater length confirms the conclusion that lengthening after contraction does not occur unless a muscle is extended by an external force.

#### METHOD

A pair of sartorius muscles of frog or toad was held by a clamp at the pelvic end in a frame similar to that used for a thermopile, the thermopile element itself being absent (see Hill 1928, p. 126; 1931, p. 271). A light chain passed up the tube from the tibial end of the preparation to a tension-recording device carried on a Palmer stand (D 12) with vertical screw adjustment. A pair of stimulating electrodes lay between the muscles very close to their pelvic end; otherwise the muscles and the chain were completely free. With the very small forces involved it was essential that the chain should hang exactly vertical, so that (1) the muscles could draw into themselves and not collapse sideways when the chain was lowered, and (2) there should be no slack to be taken up in pulling the chain straight when a tension was recorded. The thermostat, therefore, in which the muscle chamber rested, was provided with levelling screws, which were adjusted until the chain remained exactly in the middle of the tube whether it was loose or tight.

The muscles were soaked in Ringer's solution (NaCl 0.675 g., KCl 0.015 g.,  $\text{CaCl}_2$  0.020 g., phosphate-P 5.6 mg.,  $\text{H}_2\text{O}$  100 g.; pH 7.0) generally for some hours before use. Stimulation was in air, by short supermaximal condenser discharges. The opening of a single key, operating two rapid relays, provided the shock and started the sweep recording the contraction.

The tension-recorder consisted of a single  $60\mu$  nickel-chrome wire forming one arm of a bridge (Hill 1949c; see also 1949d); its resistance changed by 36 parts per million for a load of 1 g. For recording isometric contractions the bridge was connected to a very rapid galvanometer, the deflexions of which were displayed on a cathode-ray tube (Hill 1948). The current changes were so large that considerable negative feed-back was possible and lag due to the galvanometer was insignificant. For reading resting tensions a high sensitivity was required, and the bridge was connected to a Zernicke galvanometer, 1 mm. deflexion of which as used corresponded to about 10 mg. load on the wire. For reliability at high sensitivity it is better that the soldered joints at which current enters and leaves the wire should not them-



selves be strained. This was secured by leading the current in and out by short flexible copper wires soldered round the tension wire a few millimetres from each end.

#### THE LATENT PERIOD OF MUSCLE AT DIFFERENT LENGTHS

In the experiment of figure 1*A* a muscle was stimulated by several shocks at length 11 mm. (about half its natural length in the body) and made to draw up as far as it could. Isometric twitches were then recorded at the various lengths noted in the legend. The latent period clearly diminished somewhat as the length increased, but the magnification and speed were not sufficient to show how much. In figure 2*A*, on the same muscle with much higher magnification and faster sweep, it is obvious that the latent period, although slightly longer at the shorter lengths, was not much longer. If the muscle had lengthened appreciably in relaxation the latent period would have been very much greater. The observed maximum speed of shortening is given in the legend of figure 2; for the lowest two curves of figure 2*A* it was about 9 mm./sec. Had there been as much as 1 mm. of slack to be taken up the latent period would have been lengthened by 110 msec. For the record at 14 mm. the minimum allowance for taking up 1 mm. of slack would be 37 msec.; actually, the latent period was only 6 msec. longer than when the muscle was fully extended. It is clear that in none of the records of figure 2*A* is the latent period long enough for more than 0.1 to 0.2 mm. of slack to have been present. The muscle, therefore, had not lengthened appreciably in relaxation from previous shocks, even at only half its natural length.

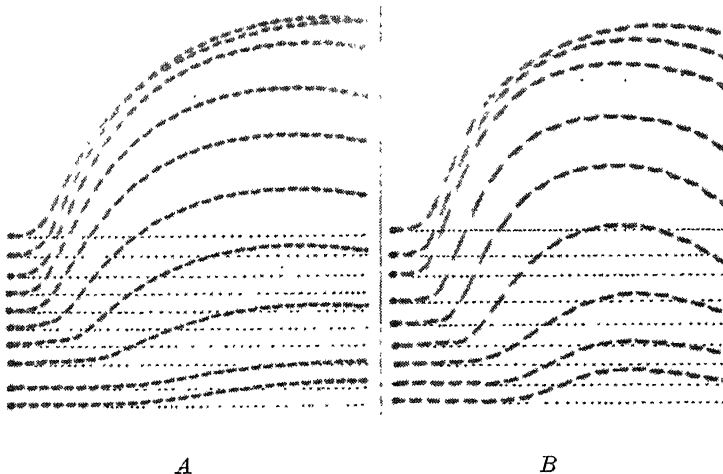


FIGURE 1. Isometric twitches at 0° C of *A*, toad's sartorii, 22.5 mm. long in body, *B*, frog's sartorii 24 mm. long in body. Time marks every 20 msec., shock at origin of trace. In *A* several preliminary shocks were given at length 11 mm., then in succession (from below upwards) twitches were recorded at lengths 11, 11, 12, 13, 14, 15, 16, 18, 20 and 22 mm. In *B*, muscles stretched to 28 mm., released to 12 mm., stretched to 15 mm., and then twitches recorded at lengths 15 mm. (1st), 15 mm. (2nd), 15 mm. (9th), 16, 17, 18, 20, 22 and 24 mm. Base-lines dotted in. In this and subsequent figures the time marks start from an arbitrary zero, not from the origin of the trace.

The experiment of figures 1*A* and 2*A* was on a toad's muscle; that of figures 1*B* and 2*B* was on a frog's. In the latter the procedure was different. The muscle was first stretched beyond its natural length, then released to 15 mm. (62%). The first, second and ninth twitches at that length were recorded, and then the muscle was stretched to various lengths and further records were made. Figure 2*B* shows the latent period with high magnification and speed. It is clear that in the first two twitches at length 15 mm. there was substantial slack to be taken up. If we assume 20 mm./sec. as the maximum speed of shortening at this length the calculated amount of slack would be about 1 mm., or in the ninth twitch about 0.5 mm. Confirming this calculation, we find that at length 16 mm. the latent period was practically as short as in the muscle fully extended; the slack at 16 mm. must have been insignificant. The muscle, therefore, had not lengthened appreciably after contracting isometrically at 16 mm., which was 67% of its natural length.

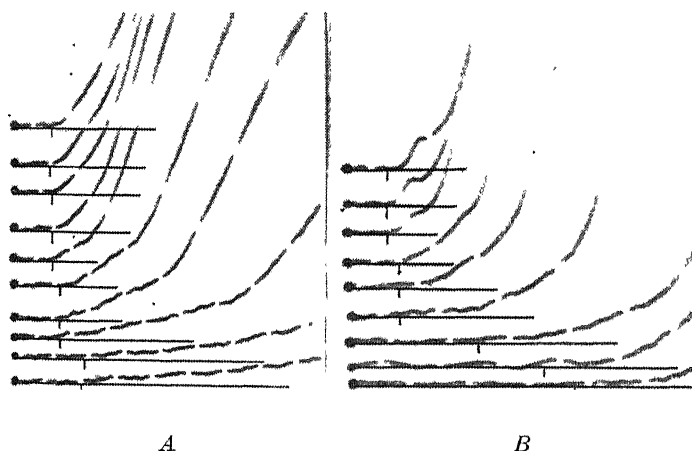


FIGURE 2. Isometric twitches, as in figure 1 but with higher speed and sensitivity to give latent period. Time marks every 20 msec., shock at origin of trace, base lines drawn in. In *A* (at 7 times the sensitivity of figure 1) the latent periods, as estimated at the vertical lines, were, in succession upwards: 41, 42, 28, 29, 24, 23, 23, 22 and 23 msec. In *B* (at 12 times the sensitivity of figure 1) the latent periods were: 75, 65, 45, 20, 20, 20, 17, 17, and 17 msec.

Note (1). The maximum speed of shortening of the toad's sartorii *A* in contracting under zero load was as follows: at initial length 16.2 mm., 34 mm./sec.; at initial length 14 mm., 27.5 mm./sec.; at initial length 11.8 mm., 9.4 mm./sec.

Note (2). The records of figure 2 were rather badly disturbed by vibration in the laboratory: those of figures 3 and 4 are better.

In the experiment of figure 3*A* a toad's muscle previously stretched was released to 14 mm. (56%) and then stimulated. Four successive isometric twitches at 14 mm. showed a large decrease of latent period, corresponding to 1 to 2 mm. of slack taken up. The fourth twitch indicated that not more than 0.2 to 0.3 mm. of slack remained. There was clearly very little if any lengthening during relaxation. In figure 3*B* the same muscle, after stretching, was released to 15 mm. Successive twitches showed about 1 mm. of slack being taken up and practically none left after the fifth shock.

In figure 3C the same muscle was first stimulated at 15 mm., then twitches were recorded at 15 mm. and various other lengths up to 25 mm. There may have been a few tenths of a millimetre slack at the shortest length.

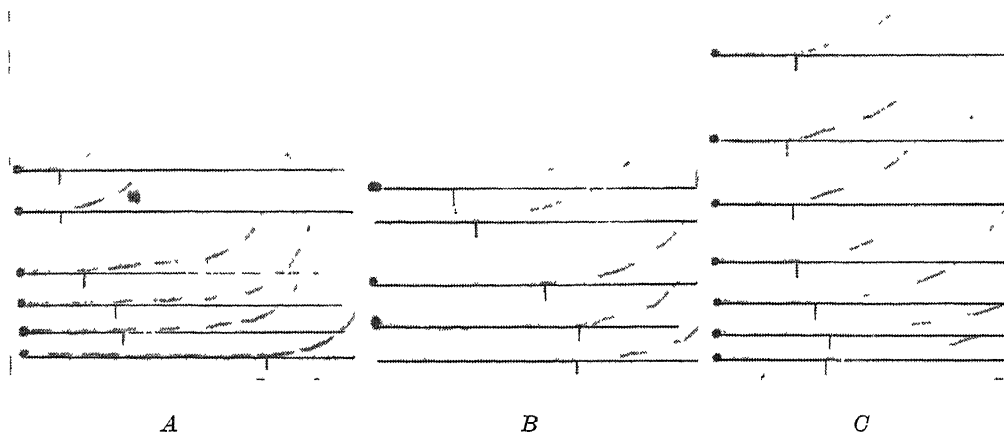


FIGURE 3. Isometric contractions at  $0^{\circ}\text{C}$  of toad's sartorii, 25 mm. long in body. High magnification to give latent period. Time marks every 20 msec., shock at origin of trace. End of latent period estimated at the vertical lines. Base-lines drawn in. *A*, stretched to 28 mm., released to 14 mm., then (successively, upwards) twitches at 14, 14, 14, 14, 16 and 18 mm.; latent periods, 110, 45, 42, 29, 19 and 19 msec. *B*, stretched to 28 mm., released to 15 mm., then five successive twitches recorded at 15 mm.; latent periods, 57, 58, 51, 30 and 24 msec. *C*, released to 15 mm. and several shocks applied; then successive twitches recorded at 15, 15, 16, 18, 20, 22 and 25 mm.; latent periods 30, 32, 27, 23, 22, 20 and 23 msec.

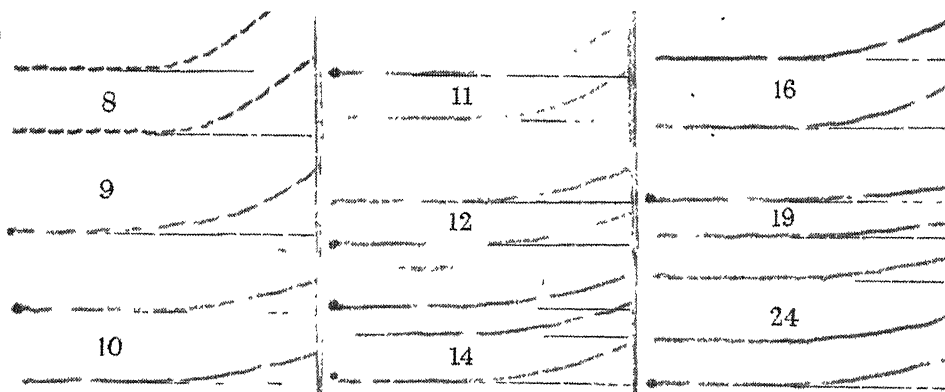


FIGURE 4. To show latent period in isometric twitch at various lengths. Toad's sartorii,  $0^{\circ}\text{C}$ , 23 mm. long in body, 59 mg. Sensitivity, 1 mm. deflexion = 0.047 g. Various speeds, time marks every 20 msec., shock at origin of trace. Base-lines ruled in. Muscles released to 8 mm. and a short tetanus applied. Twitches then recorded at the various lengths noted, in succession from 8 to 24 mm. Results as follows:

muscle length (mm.)	8	9	10	11	12	14	16	19	24
latent period (msec.)	160	48	33	24	23	23	25	29	26

In the experiment of figure 4 a pair of toad's sartorii was brought to a very short length by a brief tetanus. The latent period was then measured at various lengths, from 8 mm. (35 %) upwards. There was clearly substantial slack at 8 mm., probably of the order of 1 mm. (the maximum speed of shortening at this length would be very low), but little at 10 mm. (44 %) and beyond. It is important to note that so great a shortening had no harmful consequence and the muscle contracted normally next day.

It was possible that, even if the muscle as a whole did not show visible lengthening in relaxation, its fibres or fibrils, or even its contractile molecular filaments, might go into folds. If so, no mechanical tension could be developed between the clamp and the chain until these folds had been pulled straight, the shortness of the latent period showed that such folds were of insignificant extent. (At the speeds involved the inertia of the muscle itself is completely negligible, and any force detected is between its ends.) We must conclude, therefore, that over a wide range of lengths, rather greater in toad's muscle than in frog's, contraction is not followed by detectable lengthening during relaxation.

If the experiment be pushed to extremes, as in the records marked 8 in figure 4, a great lengthening of the latent period appears, showing that slack is actually present and that a certain amount of lengthening does occur when the muscle relaxes. It may be that at these very short lengths the weight of the muscle itself, hanging on the chain, is enough to pull it out at least at its upper (tibial) end; or that with so much shortening the circumference of the fibres, or even of the muscle as a whole, becomes so large that elastic forces either in the sarcolemma, or in the peri- or epimysium, tend to push it out again. The lengthening of the latent period, however, occurs only at lengths so short that we cannot regard it as the normal effect. Over a range of lengths considerably wider than can occur in the living body the latent period remains practically constant, showing that no significant lengthening, visible, microscopic or molecular, occurs after contraction.

#### THE TENSION-LENGTH RELATION OF RESTING MUSCLE

The tension of the recorder attached by the chain to the muscle was read to the nearest 0.005 to 0.01 g. The recorder was first raised and the muscle stretched beyond its natural length in the body. It was then lowered regularly every 1 min. in steps of 1 mm., the tension being read at each length. Successive steps caused smaller decrements of tension, until at a muscle length of about 60 to 75 % of that in the body the tension became constant. The readings were then repeated in the reverse order, the mean being taken for each length. Figure 5 shows a typical result. The final constancy of tension implied that the muscle had ceased to exert a measurable elastic pull at its lower pelvic end, near where it was held by the clamp; at its upper tibial end it was still stretched by its own weight. If the recorder were lowered much farther the muscle would begin to collapse on its clamp, and the reading would be reduced by the weight of the part so collapsed. The range, however, of constant tension was well defined experimentally, extending over 3 to 5 mm., and provided a 'base-line' for the readings at greater lengths.

The weights of the muscle and chain did not affect the result; they made up the final constant reading which was subtracted from the others. A slight complication, however, is due to the fact that the length recorded for tension  $P$  is not that of a muscle under uniform tension  $P$ , but under tension increasing from  $P$  at the pelvic (lower) end to  $(P + \text{the weight of the muscle})$  at the tibial (upper) end. This could not affect the result except at very small tensions. A further slight complication is provided by surface tension; the main effect of this also was eliminated by subtracting the final constant reading, but small variations due to it may have occurred at very low tensions. Both complications could be avoided by keeping the muscle in Ringer's solution. This was not done, since the object was to compare the resting tensions with the results of experiments on the latent period, which were made in air. For completeness, however, a few experiments were performed on muscles in Ringer's solution; the results of these were indistinguishable from the rest. For more general application of the method, e.g. in studying the possible action of chemical substances on the resting tension, it would be better to keep the muscle in Ringer's fluid throughout.

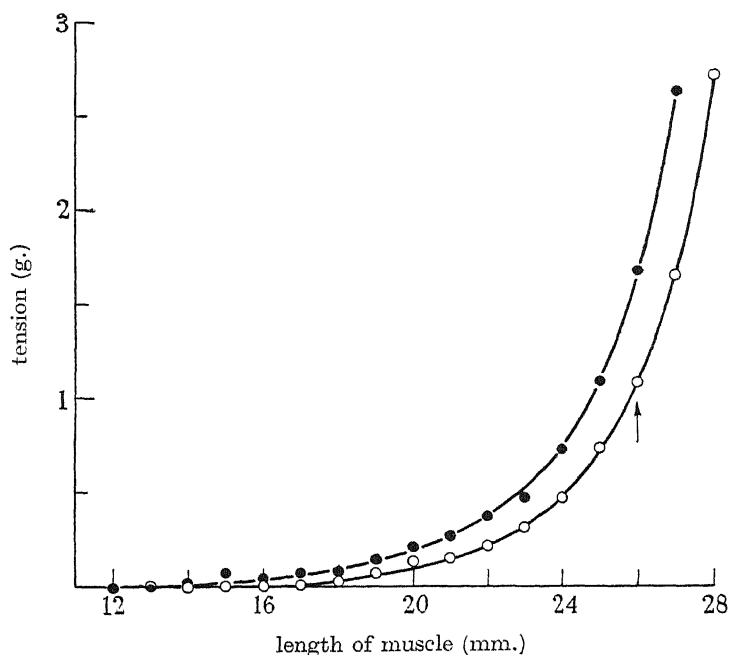


FIGURE 5. Tension-length (load-extension) curve of muscle. Toad's sartorii at 0 and 16° C. Length of muscles in body 26 mm., shown by arrow. See also A, figure 6. ●—● 16° C, ○—○ 0° C.

Nine similar series of readings of the resting tension as a function of length were made on six pairs of muscles, three from frogs and three from toads. The curves were exponential in type, the ratio of the tensions at equal increments of length being nearly constant. The results of the nine series are plotted logarithmically in figure 6,  $\log(\text{tension})$  against length. Over a wide range the relation is linear. This

form of plotting exaggerates irregularities at very low tensions, but above 50 mg. the agreement is good. It is obvious from figure 6 that the muscles were under positive measurable tension down to 60 to 75 % of their natural length.

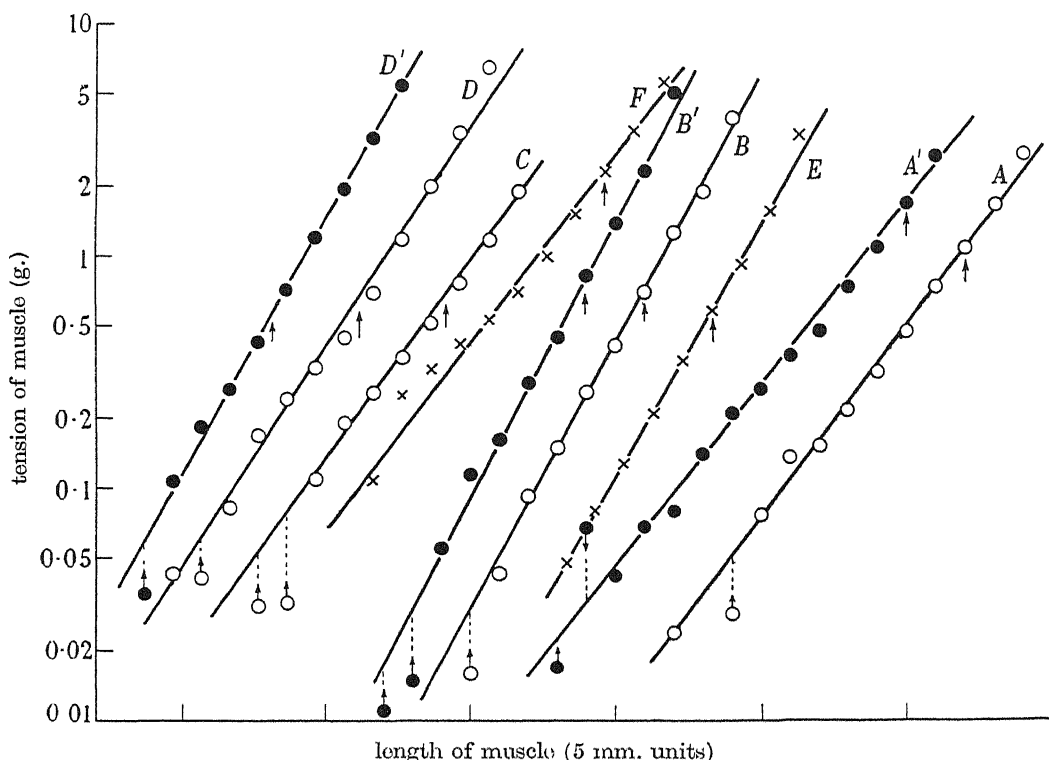


FIGURE 6. Tension-length (load-extension) curves of muscle, length on a linear, tension on a logarithmic scale. *A* and *A'*, toad's sartorii, at 0 and 16° C respectively; *B* and *B'*, toad's sartorii, at 0 and 18° C respectively; *C*, frog's sartorii at 0° C; *D* and *D'*, frog's sartorii at 0 and 18° C respectively; *E*, toad's sartorii in Ringer's solution at 9° C; *F*, frog's sartorii in Ringer's solution at 0° C. Readings of tension every 1 mm. Lengths of muscles in body, and weights: *A*, 26 mm., 60 mg.; *B*, 21 mm., 43 mg.; *C*, 26.5 mm., 116 mg.; *D*, 24.5 mm., 68 mg.; *E*, 23 mm., 47 mg.; *F*, 33 mm., 222 mg.; these lengths are shown by arrows.

The natural lengths of the muscles in the body, measured with the legs pinned out loosely in line, with the sartorii uppermost, are shown by the arrows. The resting tensions at these lengths naturally depend on the size of the muscles. Expressed in grams weight per square centimetre of muscle cross-section, the average resting tension at the natural length was  $36 (\pm 11)$  g./cm.<sup>2</sup>. This value cannot be very exact, because of the difficulty of defining the natural length precisely, but the order of quantities is certainly correct; it is about 2 to 3 % of the maximum force which the muscles could exert in a tetanus. The slopes of the lines in figure 6 appear to vary, but this is mainly due to differences of length; had length been expressed not absolutely in mm. but relatively as a fraction of the natural length the slopes would have been nearly the same. For a 10 % decrease of length the tension fell on the average by  $67 (\pm 3)$  %. No theoretical significance need be attached to

the exponential relation being length and tension; it is probably statistical in nature, depending on the presence of a large population of elastic fibres varying in length, slackness, direction, etc. As an empirical fact, however, the relation is convenient and sufficiently exact.

The muscles so far referred to had been unstimulated for a long time and stretched initially 2 or 3 mm. beyond their natural length. If a stimulus (a few shocks or a short tetanus) was applied at any length the muscle relaxed after contraction to a tension which was usually little, if at all, greater than before the stimulus. If, however, it was now stretched, its resting tension at the stretched length was found to be appreciably higher than it was at that length before the stimulus. The effect was particularly striking when both lengths were so short that, without stimulation, the resting tension was too low to measure. For example, a pair of sartorii of a large frog, natural length 33 mm., showed no measurable tension at rest at and below 23 mm. Stimulated by 10 shocks at 18 mm. and then stretched gently to 23 mm. its tension rose to 0.5 g., falling to 0.3 g. in 0.5 min.; further stretched to 28 mm., its tension rose to 2.5 g., instead of 0.5 g. as it was before the stimulus. Again, to the pair of toad's sartorii of *A*, figure 6, 6 or 8 shocks were applied at length 11 mm., and it was then gently stretched to one of the following lengths (see table) and the resting tension read from the galvanometer deflexion. It was next returned to length 11 mm., stimulated again, and then stretched to another length, etc. The second row gives the tension observed under these conditions, the third row gives what the tension had been before the muscle was stimulated.

stretched to (mm.)	14	15	16	17	18	21
tension (g.)	0.12	0.16	0.20	0.38	0.41	0.91
curve, <i>A</i> (g.) (figure 6)	—	0.017	0.024	0.035	0.052	0.160

In every case the deflexion gradually diminished, usually to about one-half. It is clear that even at very short lengths an unloaded muscle does not relax far beyond the length to which it is brought by stimulation, otherwise a tension would not be developed on raising the recorder.

The fact that the tension set up in a resting muscle, by stretching it from a length at which it has been stimulated, diminishes with time, more rapidly at first and then more slowly, led one to expect that heat would be produced equal to the difference between the mechanical work done in stretching and that recoverable in subsequent release. A few experiments were performed to verify this. The heat involved is small, and no attempt was made to obtain quantitative results. The effect, however, was obvious; the more rapid the stretch the greater was the heat. At any length a resting muscle can exert only a certain force; temporarily, however, when stretched, its resistance is greater, the muscle then 'giving' like any material stressed beyond its elastic limit.

#### DISCUSSION

Ninety years ago Kühne (1859) published the significant observation that a frog's sartorius laid on a mercury surface and stimulated did not return to its original length except when pulled out. Forty years later Kaiser (1900) repeated Kühne's

experiment with the slight variation of adding a little olive oil to the muscle on the mercury; he found the opposite effect, viz. that after contraction the muscle returned to its original form, not slowly but as though acted on by an expansive elastic force. Forty years later again Ramsey & Street (1940, 1941), working on isolated single fibres of frog's muscle, reported that such fibres held horizontally in Ringer's solution, then released by 10 to 30 % of their length and hanging in a large loop, instantly straightened on stimulation but promptly returned to the loop when the stimulus ended.

The weight of a muscle in Ringer's solution is only 5 to 6 % of its weight in air, and it can be calculated that the maximum tension in such a muscle, hanging in a loop after 20 % release, is only about 3 % of its weight in air. The tensions given in figures 5 and 6 above do not suggest that a force of 1.8 mg. could seriously extend a muscle weighing 60 mg., and the same argument applies to the single fibre. The terminal velocity of a horizontal  $80\mu$  muscle fibre falling freely in Ringer's fluid at  $20^{\circ}\text{C}$  was calculated from model experiments made on short lengths of copper wire of similar diameter. It was only 30 to  $40\mu/\text{sec.}$ , which would require about a minute for the middle of the fibre to fall 2 to 3 mm. to the vertex of its catenary. Some much more potent factor must be at work to produce the prompt return described by Ramsey & Street. They rejected the possibility that 'compressive forces in the sarcolemma' might account for the rapid lengthening after contraction. Their reason for rejection was that in the ' $\delta$ -state', which they described, a muscle remained shortened after stimulation. This argument assumes that the lateral extensibility of the fibre in the ' $\delta$ -state' is the same as normally; this will be discussed below.

An experiment was made with a toad's sartorius suspended horizontally in Ringer's solution. The pelvic bone was fixed and the tibial tendon tied to a rod adjustable horizontally. The natural length of the muscle in the body was 22.5 mm. The tibial end could be released 5.5 mm. (25 %) without visible sagging; in other words, as we should expect from the results of figures 5 and 6, the resting muscle exerted enough tension at 75 % of its natural length to maintain itself horizontal in Ringer's fluid. With 7 mm. release the sag was about 1.5 mm., with 9 mm. release about 2.5 mm. From the properties of the catenary it can be calculated that 1.5 mm. sag at the vertex implies 0.4 mm. slack, 2.5 mm. sag implies 1.2 mm. slack. Thus, the muscle released 9 mm. must have shortened altogether about 7.8 mm., which is 35 % of its length in the body. This agrees very well with the results described above. It is inconsistent, however, with the behaviour of the single fibre, which dropped into a loop as soon as released, presumably under some force much more potent than gravity since it moved so promptly, a force, moreover, which seems to be absent in the whole muscle.

'Resting length' was defined by Ramsey & Street (1940, 1941) as that at which maximum tension was developed in contraction; it was stated to be about the same as the length of the fibre when 'just taut', lying horizontally in Ringer's solution under a tension less than its weight in air. The whole muscle under such conditions is considerably shorter than its natural length in the body, and it develops its maximum force at distinctly less than its natural length (see figure 1



above). The 'resting length', therefore, cannot be taken to be the same as the natural length in the body; it may be substantially less.

The ' $\delta$ -state' described by Ramsey & Street occurred in isolated fibres of frog muscle if allowed to shorten to less than 60 to 70 % of 'resting length'. Once a fibre had passed into the ' $\delta$ -state' its mechanical properties were permanently altered; the tension developed on stimulation was much less, it no longer lengthened in relaxation but remained shortened after contraction, and the time required for the full development of tension was greatly increased.

It might be suggested that the muscles used in the present experiments were in an abnormal state caused by allowing them to shorten too much. It is true that a condition analogous to the ' $\delta$ -state' can sometimes be produced in whole muscles by continued stimulation under zero load, particularly in frogs' muscles, less readily in toads'. For example: (i) a frog's sartorius 25 mm. long in the body was stimulated at 0° C until it gave a measurable tension at length 7 mm. (28 %); it was later stretched gently to 29 mm. under a load of a few grams, after which it appeared to be irreversibly damaged, developing only a very small tension on stimulation; (ii) a toad's sartorius 22.5 mm. long was stimulated several times at 7 mm. (31 %), after which it failed to give more than a fraction of normal response and did not recover when soaked in Ringer's solution.

Apart, however, from a few such extreme cases the muscles used in the present experiments remained in constant condition for long periods. A toad's sartorius will shorten time after time to 50 % of its natural length, and give reproducible results at that length or any other; a frog's will similarly shorten to 60 %. It is difficult to believe that muscles could give such strong and consistent contractions were they in the disorganized internal condition shown by the photomicrograms of Ramsey & Street (1940, p. 30). In many experiments the results described were obtained before the muscles had been allowed to shorten very far; and in the few examples in which the ' $\delta$ -state' was found after very considerable shortening it was easily recognized. It may, therefore, be confidently assumed that the intact sartorii of English frogs and toads can shorten to 60 %, and less than 50 %, respectively, of their natural length without any sign of irreversible damage.

Consider a long cylindrical tube with a thin elastic wall; the rubber inner tube of a bicycle makes an admirable model. If the pressure in the tube is raised, its length and its diameter increase. If the elastic membrane is uniform the proportional increase in length is much less than the proportional increase in diameter; this is due (i) to the fact that at equilibrium under no external load the tension per cm. around the circumference is twice that parallel to the axis, and (ii) to the high value of Poisson's ratio, for limited extensions, in a very extensible material like rubber. If we suppose our model to be filled with contractile filaments parallel to its length, when these shorten (the volume remaining constant) the diameter increases. When they relax the tube settles down to its equilibrium length, under the pressure existing inside at rest. The question to be considered is how that equilibrium length depends on the pressure and on the elastic properties of the surface.

If the membrane is slack no resistance to shortening is met until it becomes taut; the tube can shorten without raising its internal pressure, and no force will be set

up tending to restore the initial length. As the initial pressure is raised the equilibrium length and the restoring force increase. If the elastic material of the membrane obeyed, not Hooke's law, but (like natural fibres in general) some such relation as that of figure 5 above, and if the initial pressure were low, then the restoring force would be small; with a higher initial pressure, however, the restoring force would be greater. If the tension-extension curve rose very steeply at greater extensions (as that of the sarcolemma does, parallel to the axis, according to Ramsey & Street) the restoring force would become very large and might limit the amount of shortening produced by the contractile filaments.

A contracting fibre can exert a maximum pull of about  $3.5 \text{ kg./cm.}^2$  (Ramsey & Street 1940, p. 17); it was possible that shortening might end only when the internal pressure became equal to this. Let  $T$  be the tension around the circumference and  $r$  the radius; then at the limit  $T/r = 3.5 \times 10^6 \text{ dynes/cm.}^2$  (taking  $g = 1000$  for simplicity). In an  $80\mu$  fibre, expanded to  $110\mu$  by shortening, this gives  $T = 19,000 \text{ dynes/cm.}$  According to Ramsey & Street (1940, p. 18) the resting tension of a fibre, which they believe to be borne mainly by the sarcolemma, may be as high as 80 mg. at 80 % extension. An  $80\mu$  fibre stretched by 80 % would have a diameter of  $60\mu$  and a circumference of  $190\mu$ . A load of 80 mg., therefore, would produce a tension of about 4000 dynes/cm. in the sarcolemma. This is only about one-fifth of the circular tension calculated above as required to maintain a pressure sufficient to prevent shortening. It seems likely, therefore, that before such a pressure could be reached the sarcolemma, or its internal reinforcements, would break. This may be the origin of the ' $\delta$ -state'.

We have supposed hitherto that our membrane is uniform. What happens if it is less extensible around the circumference than parallel to the axis? In our model the condition could be imitated by placing a large number of narrow rubber bands round the tube, hindering an increase of diameter but not of length. A rise of pressure then has a smaller effect on the diameter and a greater effect on the length; and the equilibrium length under a given pressure is greater. If the tube were fitted with cross-ties, reinforcing its opposition to expansion, the result would be the same. The more resistant the membrane is to lateral stretch, and the higher the initial pressure, the greater will be the equilibrium length and the restoring force.

Returning now to the ' $\delta$ -state', if we suppose (1) that in the normal fibre the visible (or other) structure provides cross-ties reinforcing the fibre against lateral expansion, and (2) that in a muscle allowed to shorten too much these cross-ties are broken or disorganized, we should expect to find that the equilibrium length, and the restoring force after contraction, would be less. Ramsey & Street's photomicrograms strongly suggest that in the ' $\delta$ -state' some such rupture of internal structure has taken place. If it has, then the fibre would be less resistant to lateral expansion and the elastic compulsion to lengthen after contraction might disappear.

If the sarcolemma were in equilibrium at rest under a circular tension of  $T$  dynes/cm. the pressure inside would be  $T/r \text{ dynes/cm.}^2$ . Let us, for illustration, take  $T$  as 20 dynes/cm. (a small enough quantity, only about one-quarter of the surface tension of water). Then the pressure in a fibre of  $80\mu$  diameter would be  $20/0.004 = 5000 \text{ dynes/cm.}^2$ , which would lead to a total extending thrust of

$5000 \times \pi(0.004)^2 = 0.25$  dyne, or about 0.25 mg., which is several times the weight of the fibre. In a muscle of 3 mm.<sup>2</sup> cross-section, such as several of those illustrated in figure 6, the extending thrust would be  $5000 \times 0.03 = 150$  dynes, or about 150 mg., sufficient to cause the muscle to lengthen several millimetres. The calculated pressure, 5000 dynes/cm.<sup>2</sup>, is about 0.005 atm., which is only one-eighth as great as the colloid osmotic pressure (30 mm. Hg) of mammalian plasma. It seems very likely that the proteins and other indiffusible substances contained in a frog's muscle fibre could maintain a pressure at least equal to this. If so, the restoring force required to extend a muscle fibre after contraction would be sufficiently accounted for.

In a whole muscle in Ringer's solution the fluid around the fibres (except those at the surface) is largely the original plasma of the animal, and the colloid osmotic pressure of the contents of the fibre would to some degree be balanced. An experiment was made on a frog's muscle left for 20 hr. at 0° C in Ringer's solution diluted to 0.64 of its usual strength. It contracted well, and the latent periods at various lengths shorter than the natural length were the same as before treatment. Apparently, therefore, the elastic extending thrust had not been significantly increased. A whole muscle, however, is a complex structure, with areolar tissue, epimysium, perimysium, blood vessels, nerve fibres, etc., closely bound together, and it might well be that these maintain a tension compensating the elastic thrust due to pressure inside the fibre. The most likely reconciliation, indeed, of the various facts, none of which is in doubt, seems to be this: that muscle fibres are normally under a small internal pressure, producing an elastic elongating force tending to make them lengthen; that in the whole muscle this elastic force is balanced by the tension existing in parallel elastic material; and that in the 'δ-state', owing to internal mechanical damage and the destruction of cross-ties, the fibre becomes much more extensible, the internal pressure falls, and the force tending to lengthen the fibre disappears.

It has been found, as will be reported in detail in a later paper (see also Hill 1949*a*), that the heat of shortening of muscle is exactly proportional to the amount of shortening over a wide range, in a toad's sartorius over as much as 50 % of the natural length. This suggests that the same identical process occurs in shortening at all lengths. It might have been expected that a given muscle shortening 1 mm. when 15 mm. long (i.e. 6.7 %) would give out more heat than in shortening 1 mm. when 30 mm. long (i.e. 3.3 %); it does not, the heat is the same.

If we suppose that the contractile molecular linkage in the fully extended muscle is similar to *A*, figure 7, and that as it shortens it changes progressively to *B*, *C* and *D*, the angles at the joints remaining constant, we can see why the heat of shortening per mm. should be the same throughout, for the active process of closing up remains identical at all stages. On this view we imagine that when stimulation ends the linkage is left (say) in state *C*, the side-arms which have folded on to each other remaining loosely attached. On gently pulling the muscle out the side-arms 'give' and allow the central zigzag to settle (say) in state *B*. In either state, *B* or *C*, the chain is just as ready to shorten when a stimulus is applied as it was in the extended condition *A*, and the latent period is the same. If the extended muscle *A*

is released without stimulation it shortens under its external elasticity, and the zigzag tends to form loose attachments at its angles, as in *B*, but not to join up as completely as when a stimulus is applied. The first stimulus after release finds a certain amount of slack and the latent period is slightly longer; for later stimuli the slack is less and the latent period diminished.

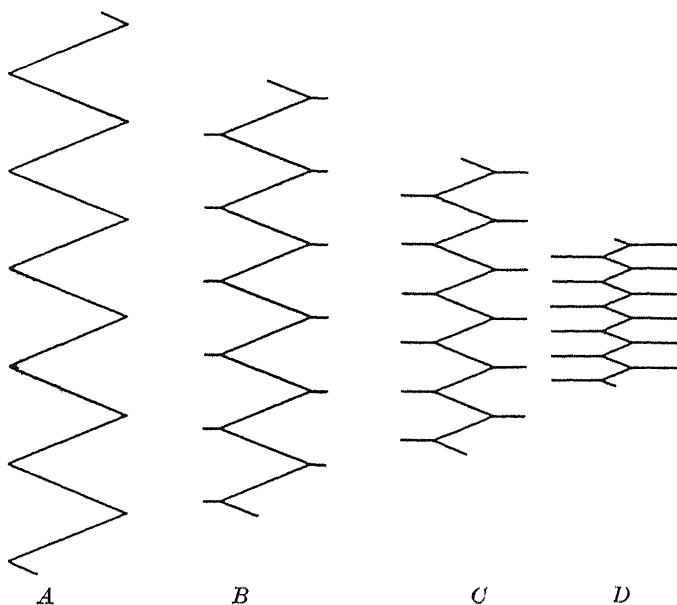


FIGURE 7. Model of contractile molecular linkage; to illustrate the constancy of the heat of shortening, the absence of lengthening in relaxation, the constancy of the latent period, and in general, the similarity of the contractile process at all lengths.

This simple model of the contractile mechanism may not conform in detail to present conclusions from X-ray diffraction studies of muscle (see, for example, Astbury 1947). It is intended, indeed, to do no more than provide a concrete picture of the pertinent facts, relating them in a generally intelligible way. If the principle behind it is correct it would be interesting to obtain X-ray diffraction pictures of living muscles at rest under a very small load, both after previous extension as in *A*, and after more or less shortening as in *B*, *C* and *D*. Considerable differences might be found. The birefringence, or the dielectric properties, of muscles in these various resting states might similarly reward investigation. Since in all these conditions at rest the properties of a muscle are the same in respect both of immediate readiness to contract (latent period) and of heat of shortening, certain likenesses would be found. If, however, the molecular linkages do fold up as much as seems to be required in a resting muscle shortened so considerably under previous stimulation, there ought to be obvious differences between the same muscle at different lengths. In every case the muscle should be at rest under zero (or very small) load; the elastic material being under small or negligible tension, any differences found could then be safely attributed to different patterns in the contractile mechanism alone.

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## The formation of choleglobin and the role of catalase in the erythrocyte

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In haemolysates of non-nucleated erythrocytes there is an inverse proportion between catalase activity and rate of choleglobin formation on addition of ascorbic acid.

In the intact erythrocytes catalase protects haemoglobin against oxidation and further destruction by the hydrogen peroxide generated by the D-amino-acid oxidase system or by physiological concentrations of ascorbic acid and glutathione.

Acid destomatization of haemolyzed horse erythrocytes causes a small decrease in the catalase activity and an increased rate of inactivation of the remaining catalase by ascorbic acid. The liberation of copper from haemocuprein is quantitatively insufficient to explain the decreased stability of the catalase.

Exposing duck oxyhaemoglobin, but not reduced haemoglobin, to a pH of 5.5 to 5.8, causes an alteration which is apparent from the increase of the rate of choleglobin formation. The mechanism of this alteration is discussed. It partly explains the 'stroma effect', at least in duck erythrocytes. In addition, in the latter, there is a true stroma effect.

Choleglobin formation in the presence of ascorbic acid is accelerated by a variety of substances. Some of these perturb haemoglobin, while others increase the formation of hydrogen peroxide from ascorbic acid.

The implications of our findings on the mechanism of choleglobin formation and on the role of catalase in the erythrocyte are discussed.

## INTRODUCTION

In 1933 Bingold suggested that the function of catalase in the erythrocyte is the protection of haemoglobin against oxidative destruction by hydrogen peroxide formed in various tissues. Catalase does, indeed, protect haemoglobin against high concentrations of hydrogen peroxide. According to Keilin & Hartree (1945) this effect is, however, indirect and is due to the shift of the equilibrium haemoglobin +

oxygen $\rightleftharpoons$ oxyhaemoglobin to the right by the oxygen formed on catalatic decomposition of hydrogen peroxide. Oxygenated haemoglobin is less readily oxidized by hydrogen peroxide than is reduced haemoglobin. If, on the other hand, low concentrations of hydrogen peroxide are produced in the system by the action of notatin on glucose, Keilin & Hartree (1945) found that the catalase in the erythrocyte does not prevent oxidation of haemoglobin to methaemoglobin. They therefore concluded that the function of catalase in the erythrocyte is not predominantly a protective one.

The participation of hydrogen peroxide in the coupled oxidation of ascorbic acid and haemoglobin has been demonstrated by Lemberg, Legge & Lockwood (1939); cf. also Engel (1940). Lemberg, Cortis-Jones & Norrie (1938*b*) had similarly shown that catalase partly inhibits the coupled oxidation of ascorbic acid and pyridine haemochromogen. It seems likely, therefore, that in the erythrocyte, catalase should also be able to protect haemoglobin against the action of the rather high physiological concentrations of reducing substances such as ascorbic acid and glutathione. In this paper we show that catalase protects haemoglobin against oxidation to choleglobin in the mammalian erythrocyte.

Lemberg, Legge & Lockwood (1941) presented evidence that the stroma of the erythrocyte contains a protective factor which inhibits the coupled oxidation of ascorbic acid and haemoglobin. On removal of the stroma by precipitation at pH 5.8, a marked increase in choleglobin formation was observed. We have now confirmed this observation and have shown that this so-called stroma factor consists of several distinct effects. First, we have shown that acid destromatization affects the rate of choleglobin formation indirectly, by decreasing catalase activity by reducing the stability of catalase in the presence of ascorbic acid in the haemolysate, and by removal of some catalase in the precipitated stroma. Secondly, oxyhaemoglobin undergoes changes at pH 5.5 to 5.8, which cause an increase in the rate of its coupled oxidation with ascorbic acid. Lastly, at least in nucleated erythrocytes, there is some evidence that the stromatic or nuclear material exerts a direct protective influence on haemoglobin.

#### MATERIALS AND METHODS

The catalase used in these experiments was prepared from horse erythrocytes. Fresh cells were washed and haemolyzed with toluene and water. The haemoglobin was denatured and precipitated with alcohol and chloroform as described by Agner (1942). It was found possible to remove haemoglobin practically completely by allowing the alcohol-chloroform solution to stand overnight before separating the precipitate. Inert protein was then removed by precipitation at pH 5.3, followed by adsorption of the catalase on alumina. After elution with dilute ammonia catalase was precipitated at 60 % saturation with ammonium sulphate and dialyzed. The final preparation had a Kat.-F. of 56,000. Catalase activities were determined by the method of Zeile & Hellström (1930).

Erythrocytes of all species (horse, duck, chicken) were spun down, washed once with physiological saline as soon as possible after collection and stored at 0° C.

Crystalline oxyhaemoglobin was prepared from horse erythrocytes by the method of Heidelberger (1922). It was freed of catalase by treatment with alumina at pH 5.5. The haemoglobin solutions were incubated with ascorbic acid in thin layers in 100 ml. Erlenmeyer flasks to ensure complete oxygenation. Choleglobin concentration was estimated by the absorption of the CO-haemochromogen using a Spekker absorptiometer with red filter H558 no. 608. By a comparison with the spectrophotometric method (cf. Lemberg *et al.* 1941), it was found that up to a concentration of about 50 % choleglobin the increase of the absorption measured in the Spekker was proportional to the choleglobin concentration. The choleglobin percentages approximately corresponding to the figures read on the Spekker absorptiometer are given in the legends of the figures.

Glutathione was estimated by the method of Quensel & Wachholder (1934). Ascorbic acid was estimated by titration either with 2:6-dichlorophenolindophenol or with iodine after precipitation of proteins by the addition of trichloroacetic acid to the carbon monoxide saturated solution. Stock solutions of ascorbic acid were made up freshly every few days. They were neutralized to pH 6.0 and kept under paraffin oil. Copper was estimated colorimetrically by the method of Mann & Keilin (1938) (cf. Foulkes & Lemberg 1948). An acetone powder of pig's kidney was used as source of D-amino-acid oxidase.

## EXPERIMENTAL

### (1) *The role of catalase*

The catalase protection of haemoglobin against irreversible coupled oxidation with ascorbic acid is well illustrated in figure 1. In the absence of catalase crystalline horse oxyhaemoglobin (curve *A*) is rapidly destroyed; there is practically no initial lag. A catalase-free haemolysate of stored duck erythrocytes (curve *B*) shows only a short lag. The lag terminates rather abruptly in a manner which seems to be characteristic of duck haemoglobin. The maximum rate of choleglobin formation is similar in curves *A* and *B*. Curve *D* shows the rate of choleglobin formation with haemolyzed horse cells containing their original catalase ( $7 \times 10^{-8}$  M final concentration). Curve *C* was obtained with the same oxyhaemoglobin solution as curve *A*, to which was added pure erythrocyte catalase to the same final concentration as in curve *D*. These results show that catalase inhibits choleglobin formation by prolonging the lag period. That catalase can protect haemoglobin against the action of more physiological concentrations of autoxidizable reducing substances is shown by the following experiment: 0.5 ml. washed horse cells + 1 ml. 0.2 M-phosphate buffer of pH 7.2 + 0.25 ml. 2 % glutathione + 0.05 ml. 2 % ascorbic acid were mixed in 100 ml. Erlenmeyer flasks. To one, azide was added to a final concentration of 0.01 M. The volumes were made up to 5 ml. with saline. The flasks were incubated at 37° C. While even after 6 hr. no spectroscopic changes could be observed in the absence of azide, in the presence of azide already after 2 hr. large amounts of methaemoglobin had been formed, and after 6 hr. significant amounts of choleglobin were observed. The cells were intact at the end of the experiment. Qualitatively similar results were obtained when ascorbic acid and glutathione were used in

concentrations of 1 and 40 mg. % respectively. Unlike the results of Keilin & Hartree with notatin, our experiments with D-amino-acid oxidase show that catalase does protect haemoglobin against the hydrogen peroxide formed by this system. 0.5 ml. washed cells + 0.5 ml. 2 % dl-methionine + 1 ml. oxidase in 0.06 M-phosphate buffer of pH 7.3 + saline to 5 ml. were incubated at 37° C in presence or absence of azide. Control tubes were also set up with cells and saline but omitting in turn methionine, oxidase and azide. Only in the presence of all these three components did the haemoglobin of the intact cells undergo any alteration; under these conditions it was almost completely transformed into methaemoglobin and choleglobin after 1 hr. Keilin & Hartree (1946) have shown that only undissociated azide permeates into the erythrocyte; little azide will thus penetrate at pH 7.3, apparently sufficient to inhibit intracellular catalase but not sufficient to give appreciable amounts of azide methaemoglobin under our experimental conditions.

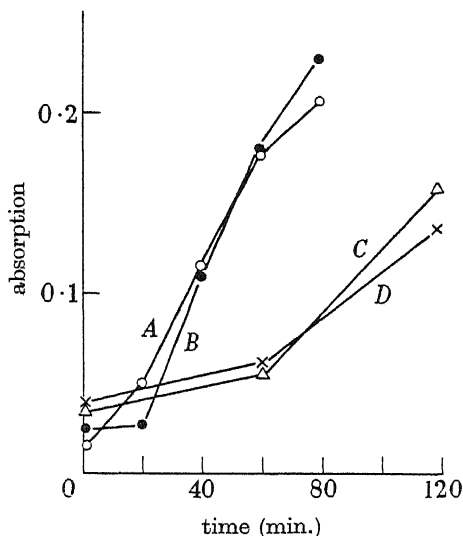


FIGURE 1. Catalase activity and choleglobin formation. Haemoglobin 0.6 g. %; ascorbic acid 180 mg. %; phosphate 0.024M pH 6.8. Temp. 37° C. Curve A, crystalline horse oxyhaemoglobin freed from catalase. Curve B, duck haemolysate. Curve C, crystalline horse oxyhaemoglobin + catalase. Curve D, horse haemolysate. Ordinate 0.1 = 24 % and 0.2 = 51 % choleglobin respectively.

## (2) *The stroma factor*

Lemberg *et al.* (1941) found that destromatization at pH 5.8 and subsequent neutralization has a significant effect on choleglobin production. This was attributed to some factor in the stromatic material. Such a factor does indeed occur in the nucleated erythrocytes of the duck.

A haemolysate of duck cells contains a heavy flocculate of nuclear and stromatic material. In a haemolysate containing 1 g. % haemoglobin and 200 mg. % ascorbic acid, the removal of this flocculate by centrifuging at neutral pH decreased the lag in choleglobin formation at 37° C from 70 to 30 min.



No sedimentation occurs on centrifuging of a haemolysate of horse erythrocytes at 2500 r.p.m. at neutral pH. In an attempt to establish whether the stroma of horse erythrocytes also contains a protective factor, or whether the so-called stroma effect is an artefact due to the acidification of the haemolysate, destromatization was carried out with toluene (Heidelberger 1922). Toluene destromatization led to a variable increase in the rate of choleglobin formation as compared to the behaviour of a non-destromatized control. Even if the stroma is not removed, however, toluene accelerates choleglobin formation: 0.5 ml. horse erythrocytes were mixed with 0.1 ml. toluene and allowed to stand for  $\frac{1}{2}$  hr. at room temperature. 8 ml. distilled water, 0.5 ml. of 0.6M-phosphate buffer of pH 6.8, and 1 ml. 2% ascorbic acid, all at 37° C, were then added, and the mixture was incubated in a 100 ml. Erlenmeyer flask at 37° C. There was a lag in choleglobin formation of about 20 min. under these conditions. The lag in a control flask, in the absence of toluene, was almost 2 hr.

As a similar result was obtained with a sample of crystalline horse oxyhaemoglobin practically free of catalase, toluene must act on the oxyhaemoglobin directly.

No information could thus be gained on the influence of the stroma itself on the formation of choleglobin in haemolysates of non-nucleated erythrocytes.

### (3) *The effect of acid destromatization on catalase*

The protective role of catalase is also concerned with the action of the 'stroma factor' described by Lemberg *et al.* (1941). The relation between catalase and the effect of destromatization at pH 5.5 is illustrated by the results of a typical experiment described in figure 2, in which relative choleglobin concentrations and catalase activities were measured simultaneously. It is obvious that in the presence of stroma there is far less choleglobin formation than when the stroma has been removed by centrifugation at pH 5.5 to 5.8, followed by neutralization. The destromatization affects catalase activities in two ways: (a) there is a slight loss of catalase, about 20% of which could be recovered on suspending the centrifuged stroma in alkaline phosphate. (b) In the destromatized haemolysate there is a marked increase in the rate of inactivation of catalase during the action of ascorbic acid and oxygen. Added pure catalase (curve C) is also rapidly inactivated. Figure 2 also illustrates the inverse relationship between catalase concentration and rate of choleglobin formation shown in figure 1. Acid destromatization thus either removes a substance which protects catalase against ascorbic acid, or else liberates a factor which increases the inactivation of catalase by ascorbic acid. We have previously shown (Foulkes & Lemberg 1948) that ionic copper is required for the ascorbic acid inhibition of catalase. The copper protein present in erythrocytes, haemocuprein, like other copper proteins, is split by acids with liberation of ionic copper (Mann & Keilin 1938). The lower the pH, the more copper is split from the copper proteins (Boyden & Potter 1937). In fact, we found that the lower the pH of destromatization, the more pronounced was the increase in the rate of choleglobin formation (see figure 3). We accordingly tested the hypothesis that the effect of acid destromatization on the rate of choleglobin formation is simply due to liberation of copper on acidification.

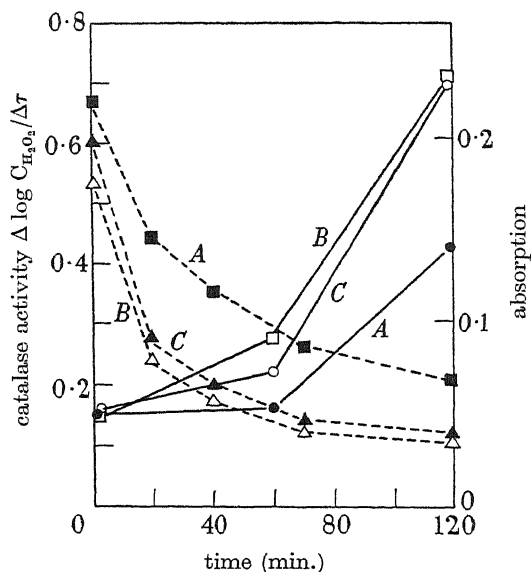


FIGURE 2. Stroma effect and catalase activities. Horse haemolysate, 1.2 g. % haemoglobin; ascorbic acid 150 mg.%; phosphate 0.03M pH 6.8. Temp. 37° C. Full lines: light absorption; interrupted lines: catalase activities. A, horse haemolysate. B, horse haemolysate after acid destromatization. C, acid destromatized horse haemolysate + catalase. Right-hand ordinate 0.1 = 12 % and 0.2 = 26 % choleglobin respectively.

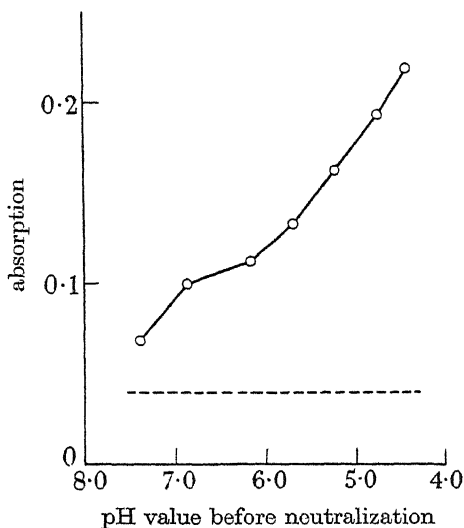


FIGURE 3. Effect of pH of destromatization. Horse haemolysate, 1.0 g. % haemoglobin; ascorbic acid 100 mg.%; phosphate 0.03M pH 6.8. Temp. 37° C. Light absorption at time 0—interrupted line; after 2 hr.—full line. Ordinate 0.1 = 14 % and 0.2 = 31 % choleglobin respectively.

(i) Addition of  $\text{Cu}^{++}$  to a final concentration of  $1\text{ }\mu\text{g./ml.}$  to a horse-cell haemolysate produced an effect which is essentially the same as the effect of acid destromatization. This is easily seen from figure 4.

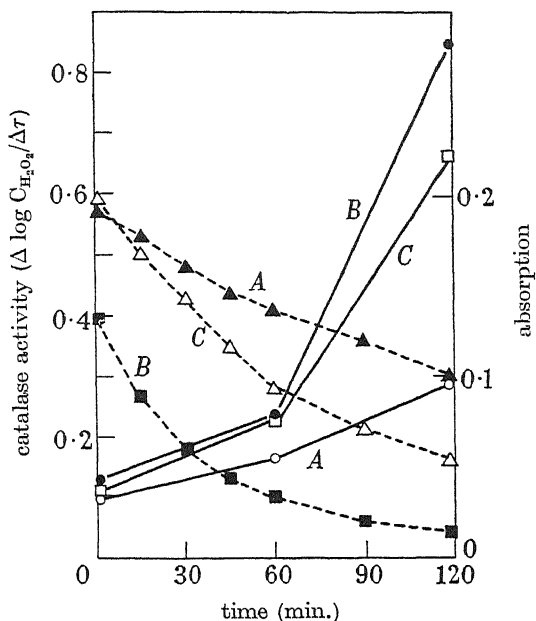


FIGURE 4. Influence of copper on choleglobin formation. Horse haemolysate, haemoglobin  $1.2\text{ g. \%}$ ; ascorbic acid  $150\text{ mg. \%}$ ; phosphate  $0.025\text{ M}$  pH  $6.8$ . Temp.  $37^\circ\text{ C}$ . Full lines—light absorption; interrupted lines—catalase activities. A, horse haemolysate; B, acid destromatized horse haemolysate; C, horse haemolysate +  $1.6 \times 10^{-5}\text{ M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $1\text{ }\mu\text{g./ml. Cu}$ ). Right-hand ordinate  $0.1 = 12\text{ \%}$  and  $0.2 = 26\text{ \%}$  choleglobin respectively.

(ii) Estimation of the amount of copper set free by destromatization was attempted in various ways. The most consistent results were obtained by the following method:  $10$  to  $50\text{ ml.}$  washed erythrocytes were haemolyzed with the minimum amount of double glass distilled water. The haemolysate was transferred quantitatively into sausage casings (Tee-Pak). These had previously been treated with  $1\text{ \% (v/v)}$  sulphuric acid and distilled water till free from copper. The bag contents were then dialyzed for  $3$  to  $5$  days at  $4^\circ\text{ C}$  against the twentyfold volume of copper-free hydrochloric acid of a concentration such that the final pH of the bag contents became the same as the pH of destromatization ( $5.5$  to  $5.8$ ). The concentration of hydrochloric acid usually required was of the order of  $\text{M}/80$  to  $\text{M}/100$ . On completion of the dialysis the dialysate was evaporated down, then digested with sulphuric acid and freshly distilled hydrogen peroxide. The copper was then estimated in the usual way. In some experiments the copper of the bag contents after dialysis was also estimated. The overall results of many experiments show that in  $1\text{ ml.}$  of either horse or duck cells  $0.2$  to  $0.4\text{ }\mu\text{g.}$  copper becomes dialyzable at pH  $5.5$  to  $5.8$ .

The final concentration of added copper, required to produce an effect similar to that produced by acid destromatization, is of the order of  $1\text{ }\mu\text{g./ml.}$  (see figure 4).

Much smaller effects are produced by smaller additions of copper. As the final dilution of the erythrocytes in these experiments was about 1 in 20, 20  $\mu\text{g.}$  copper would have to be liberated from 1 ml. of erythrocytes on acidification to pH 5.5 to 5.8, in order to account for the effect of acid destromatization. The total concentration of copper in horse (and duck) erythrocytes is, however, only 1 to 2  $\mu\text{g./ml.}$

It is thus clear that the liberation of copper from haemocuprein is quantitatively insufficient to account for the high rate of choleglobin formation in acid destromatized haemolysates. The protection of catalase in the undestromatized haemolysate may be a true stroma effect.

(4) *The effect of acid destromatization on haemoglobin*

In addition to the previously described action on the catalase activity in haemolysates, acid destromatization also has a direct effect on oxyhaemoglobin. This effect was revealed when experiments were carried out with duck erythrocytes, which do not contain catalase. The same experiments carried out with haemolyzed horse cells revealed no clear-cut difference between the effect of aerobic and anaerobic destromatization (cf. below).

Freshly collected duck erythrocytes were haemolyzed and the heavy flocculate of stroma and nuclear material was spun off at neutral pH (cf. § 2). The slightly cloudy haemolysate thus obtained was divided into several fractions which were used for the following experiments:

(A) The haemolysate was acidified to pH 5.5 in the presence of air; a small precipitate which formed was removed by spinning. The pH was then adjusted to 6.8, ascorbic acid was added and the rate of choleglobin formation was determined in the usual way.

(B) Before acidification, the haemolysate was thoroughly deoxygenated in a high vacuum. The acidified haemolysate was rapidly spun down under paraffin oil and finally neutralized before appreciable oxygenation had taken place. It was then divided into two parts. In the first ( $B_1$ ) choleglobin formation was determined directly. The second one ( $B_2$ ) was acidified to pH 5.5 in the presence of oxygen. After short standing followed by neutralization, ascorbic acid was added, and the rate of choleglobin formation again determined.

(C) Untreated haemolysate served as control.

It can be seen from figure 5 that oxyhaemoglobin, but not reduced haemoglobin, undergoes on acidification an alteration, either with formation of a more labile form of haemoglobin which reacts faster with ascorbic acid or with formation of an intermediate catalyst which speeds up the reaction. Either of these hypotheses would explain the lag in the formation of choleglobin on incubating haemoglobin with ascorbic acid, and also the fact that this lag closely corresponds to the lag in the disappearance of ascorbic acid. This is shown by experiments in which the duration of the lag was decreased by the addition of copper (cf. figure 6). Similar results were obtained when the lag was prolonged by the addition of catalase.

Addition of alkaline-denatured haemoglobin decreases the lag period. This is illustrated in table 1. It is seen that whilst in high concentration even native proteins greatly reduce the lag in choleglobin formation, in low concentration this

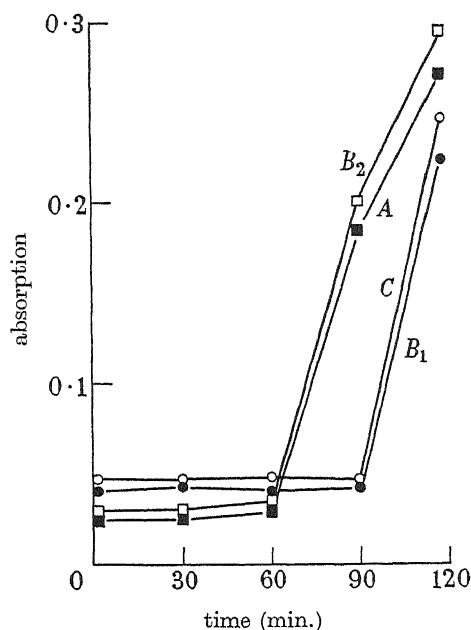


FIGURE 5. Effect of acidification of oxyhaemoglobin on choleglobin formation. Centrifuged duck haemolysate, haemoglobin 1 g. %; ascorbic acid 150 mg. %; phosphate 0.033 M pH 6.8. Temp. 37° C. *A*, acid destomatized in air; *B*<sub>1</sub>, acid destomatized in a high vacuum; *B*<sub>2</sub>, acid destomatized in a high vacuum, then acidified in air; *C*, non-destomatized control. Ordinate 0.1 = 14 % and 0.2 = 31 % choleglobin respectively.

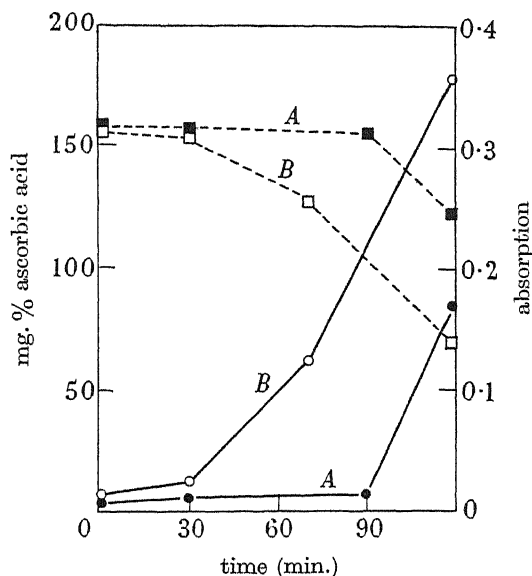


FIGURE 6. Lag in choleglobin formation and ascorbic acid disappearance. Centrifuged duck haemolysate, haemoglobin 1 g. %; phosphate 0.025 M pH 7.3. Temp. 37° C. Full lines—light absorption; interrupted lines—ascorbic acid concentration. *A*, control; *B*, control +  $10^{-4}$  M- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . Right-hand ordinate 0.1 = 14 %, 0.2 = 31 % and 0.3 = 48 % choleglobin respectively.

effect is specific for denatured haemoglobin. Denatured globin, prepared by splitting oxyhaemoglobin with acetone-HCl and dissolving the precipitated protein in cold alkali (pH 13), had no effect. Proteins boiled in alkali were found to increase the rate of choleglobin formation even in small concentration; this cannot, however, explain the effect of denatured globin haemochromogen. The action of pyridine might also be explained by the formation of a trace of haemochromogen.

TABLE 1. Specific and unspecific influences on the rate of choleglobin formation at 37° C

species etc.	haemo- globin (g. %)	ascorbic acid (mg. %)	phosphate buffer pH 6.8 (M)	treatment	lag (min.)
horse haemolysate	1.0	200	0.025	control	80
				+ 0.02 g. % haemoglobin denatured with cold alkali	40
				+ 0.02 g. % haemoglobin denatured with hot alkali	30
				+ 0.02 g. % globin denatured with cold alkali	80
				+ 0.02 g. % globin denatured with hot alkali	50
				+ 0.02 g. % egg albumen denatured with hot alkali	50
	1.5	100	0.011	after acid destromatization	40
				control	120
	1.0	200	0.25	+ 1 % (v/v) pyridine	10
			0.025	—	15
crystalline horse oxyhaemoglobin free from catalase	1.7	160	0.025	copper free	80
				copper free	
				control	15
				+ 0.6 g. % horse serum proteins	0
				+ 0.6 g. % serum albumen	0
duck haemolysate	1.0	150	0.033	control	50
				+ 0.5 g. % copper-free gelatine	30

It was also observed that on acid destromatization there invariably occurred in the acidified solution some oxidation of haemoglobin to methaemoglobin. However, in confirmation of the results of Lemberg *et al.* (1941) that methaemoglobin is not transformed to choleglobin at a more rapid rate than haemoglobin, we found that choleglobin formation was independent of the amount of methaemoglobin formed during acid destromatization.

Haematin decreases the lag in choleglobin formation from haemoglobin in the presence of ascorbic acid. It was found, however, that concentrations of haematin of the order of 5 to 10 mg. % were required to produce a significant effect on the rate of choleglobin formation in a haemolysate of human erythrocytes (1 g. % haemoglobin, 200 mg. % ascorbic acid), whereas very little haematin, if any, would be produced from oxyhaemoglobin at pH 5.5.

(5) *Effect of diethyldithiocarbamate on choleglobin formation*

A direct reaction with haemoglobin seems also to explain the action of diethyldithiocarbamate on the coupled oxidation of haemoglobin and ascorbic acid. Lemberg *et al.* (1939) showed that diethyldithiocarbamate, although practically completely abolishing the formation of hydrogen peroxide from autoxidation of ascorbic acid, did not reduce the rate of choleglobin formation from haemoglobin. Inspection of their results reveals that diethyldithiocarbamate even increased the oxygen uptake. We have confirmed these results. In the presence of ascorbic acid diethyldithiocarbamate causes a large increase in the rate of choleglobin formation. In the absence of ascorbic acid, but in the presence of hydrogen peroxide, diethyldithiocarbamate also accelerates oxidation of haemoglobin, mainly to methaemoglobin (figure 7): 6.25 ml. of a destromatized haemolysate of duck erythrocytes buffered at pH 6.8 were put at room temperature into the cup of a Spekker photo-

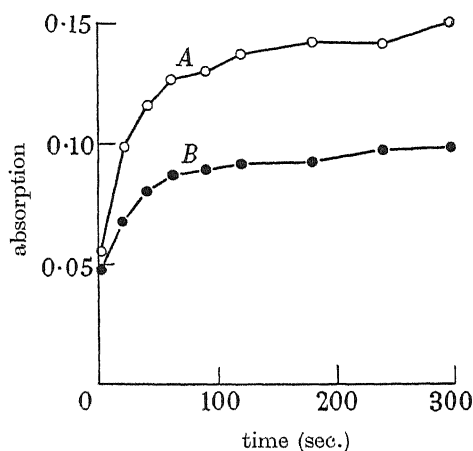


FIGURE 7. Action of diethyldithiocarbamate on the oxidation of haemoglobin by hydrogen peroxide. Centrifuged duck haemolysate, haemoglobin 0.65 g.%; hydrogen peroxide  $5.6 \times 10^{-5} \text{ M}$ ; phosphate 0.03 M pH 6.8. Temp.  $25^{\circ} \text{C}$ . A,  $4 \times 10^{-3} \text{ M}$  diethyldithiocarbamate; B, control. Ordinate 0.1 = 7% and 0.15 = 14% methaemoglobin respectively.

electric colorimeter. At time 0, hydrogen peroxide was added to a final concentration of  $5.6 \times 10^{-5} \text{ M}$ , and the rate of oxidation of haemoglobin was measured by the increase of absorption above  $600 \text{ m}\mu$ . Figure 7 shows that in the presence of diethyldithiocarbamate the rate of oxidation is higher than in its absence. No significant amounts of choleglobin were formed. On the other hand, methaemoglobin is reduced by diethyldithiocarbamate (Keilin & Hartree, 1946). In the absence of ascorbic acid, oxyhaemoglobin is little affected by diethyldithiocarbamate. Traces of methaemoglobin only are formed. There is thus no large production of hydrogen peroxide under these conditions. As formation of hydrogen peroxide from ascorbic acid is inhibited by diethyldithiocarbamate, these experiments do not invalidate the evidence of Lemberg *et al.* (1939) that, at least in the initial stages, choleglobin formation occurs under conditions excluding the formation of free hydrogen peroxide.

## DISCUSSION

Our experiments have shown that acid destromatization can affect the rate of choleglobin formation in three different ways. In the mammalian cell it was not possible to determine quantitatively the relative contribution of the various factors to the total effect of acid destromatization. First of all there is the effect observed on centrifuging haemolysates of duck erythrocytes. Secondly, in the mammalian cells which contain catalase, acid destromatization greatly affects the catalase activities of the haemolysates in the presence of ascorbic acid. Thirdly, oxyhaemoglobin, but not reduced haemoglobin, undergoes some change on acidification to pH 5.5 to 5.8, which leads to a decreased lag in the choleglobin formation. This could be proved for duck oxyhaemoglobin, but probably the same holds for horse oxyhaemoglobin. Vladimirov & Kolotilova (1947) appear to have made similar observations. In this connexion it is interesting to note that Liébecq, Delbrouck & Prijot (1947) observed that slight acidification to a pH at which methaemoglobin, but no acid haematin, is formed, liberated a large percentage of the iron of oxyhaemoglobin but not of carboxyhaemoglobin or methaemoglobin.

The action of various substances such as pyridine, toluene, high concentrations of phosphates or various proteins, diethyldithiocarbamate, as well as partly that of copper (Kiese 1947), may consist in 'perturbation' (Holden 1947) of the oxyhaemoglobin. Perturbation of the protein, by the above substances, by acidification, or during the initial phases of the reaction of oxyhaemoglobin with ascorbic acid, activates the haem-bound oxygen, and at the same time may bring it into closer contact with hydrogen donor groups in the protein; this leads to oxidative changes in the protein. When this oxidatively altered haemoglobin is formed, the reaction is speeded up. As we have seen, intact oxyhaemoglobin reacts only slowly with ascorbic acid, whereas the oxygen of strongly acidified oxyhaemoglobin reacts instantaneously and forms hydrogen peroxide (Lemberg 1942). Similarly, the oxygen of the oxidative perturbation product of oxyhaemoglobin may also react at an increased rate with ascorbic acid. According to Lemberg *et al.* (1939) the intramolecular decomposition of the hydrogen peroxide-haem complex causes the oxidation of the prosthetic group.

The effect of catalase shows that this complex is dissociable. It is then possible that during the initial stages of the coupled oxidation a peroxidative catalyst is formed which acts on haemoglobin, oxidizing its prosthetic group. This hypothesis is supported by the effect of small amounts of alkali-denatured haemoglobin on the rate of choleglobin formation.

The formation of a pseudo-peroxidative catalyst of haemochromogen nature has been postulated by Granick & Gilder (1947) as explanation of irreversible haemoglobin destruction *in vivo*. They assumed that this catalyst forms a hydrogen peroxide complex which reacts with haemoglobin, transforming the latter into denatured globin haemochromogen and being itself destroyed in the process with the oxidation of the prosthetic group. This scheme fails to explain the overcoming of the initial lag phase in our experiments, nor is it in agreement with the behaviour of denatured globin haemochromogen *in vitro*. The latter reacts only very slowly with ascorbic acid and oxygen, yielding verdohaemochromogen, not a cholehaematin



derivative (Lemberg *et al.* 1938a). Moreover, choleglobin, the product of the coupled oxidation of haemoglobin and ascorbic acid, still possesses a native, though presumably altered, protein, quite different from the protein of alkali-denatured haemoglobin which in our experiments acts as catalyst. It is also doubtful whether hydrogen peroxide can be formed by autoxidation of haemochromogens, whereas it is formed by coupled oxidation. The catalytic action of the haemochromogen can, therefore, be only due to a true peroxidative activity without valency change of the iron.

The action of copper in increasing the coupled oxidation of haemoglobin and ascorbic acid may also be partly due to increased formation of hydrogen peroxide from ascorbic acid. Other reagents, which directly or indirectly lead to formation of hydrogen peroxide, also accelerate choleglobin formation, e.g. 7-amino-8-hydroxyquinoline (cf. Albert & Falk 1949). The stimulation of oxygen uptake found by Stoltz, Harrer, Schultze & King (1937) on addition of 8-hydroxyquinoline to washed liver brei in the presence of ascorbate and haemolyzed blood also appears to be due to such an effect (Albert & Falk 1949). The acceleration of choleglobin formation in high concentrations of phosphate may also be due to the decreased stability of ascorbic acid in the presence of phosphate (Cavallini 1945).

The failure of catalase to protect haemoglobin in the erythrocyte against the hydrogen peroxide formed by notatin and glucose (Keilin & Hartree 1945) is difficult to explain. Catalase afforded protection against hydrogen peroxide formed by D-amino-acid oxidase or by ascorbic acid and glutathione. Whatever the explanation may prove to be, the failure of catalase to protect haemoglobin against hydrogen peroxide formed by notatin is certainly not a general phenomenon. An interesting speculation further arises from our finding of the protective action of catalase. The nucleated erythrocytes of the duck contain practically no catalase. Chicken erythrocytes have a slight catalase activity but considerably less than mammalian cells. The comparative amounts of catalase activity in these three species were: horse, 1500; chicken, 86; and duck, 4. On the other hand, we found that erythrocytes from a duck contained ascorbic acid (2 mg. %) and reduced glutathione (54 mg. %) in the same concentrations as mammalian erythrocytes. If the average lifetime of the duck erythrocyte is of the same order as that found for hen erythrocytes by Hevesey & Ottesen (1945), the short lifetime of these avian nucleated erythrocytes as compared with that of the mammalian catalase-containing cells may perhaps be partly explained by the fact that they contain no catalase to protect oxyhaemoglobin against the action of ascorbic acid and glutathione.

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## The influence of nerve fibres on Schwann cell migration investigated in tissue culture

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[Plate 19]

A study of tissue cultures prepared from rabbit sciatic nerves was undertaken to determine the effect of reinnervation on the amount of cell outwandering from nerves undergoing Wallerian degeneration. For this purpose, 25 and 100 days before culturing, some nerves were completely severed and reinnervation prevented, while others were crushed, allowing rapid reinnervation. It was found that reinnervation profoundly diminished the amount of Schwann cell outwandering, in comparison with the outwandering from non-reinnervated nerves. This effect extended only a few cm. distally to the lesion when the nerve was cultured 25 days after interruption, but by 100 days extended much farther towards the periphery. In the region a few cm. distally to the lesion the intensity of the effect was little if at all increased by prolonging the period between operation and culture. The effect was confirmed in reinnervated nerves prepared by complete severance and subsequent suture. Reinnervation influences only the Schwann cells in this way; the outwandering of cells other than Schwann cells did not differ significantly between reinnervated and non-reinnervated nerves. The total cell population within the nerves, estimated from histological sections, is not detectably affected by reinnervation. The interpretation suggested is that a specific adhesion develops between Schwann cells and nerve fibres, and this prevents the migration during tissue culture of a proportion of the Schwann cells.

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## INTRODUCTION

A previous paper (Abercrombie & Johnson 1942) gave an account of the outgrowth of cells from fragments of rabbit sciatic nerve cultivated *in vitro*. It was shown that normal, intact nerve gave rise to almost no outgrowth in the standardized conditions of cultivation used. When, however, explants were taken from the stumps of nerves severed at a previous operation, then outgrowth of Schwann cells, fibroblasts and macrophages occurred, as Ingebrigtsen (1916) had previously observed. Analysis was concentrated mainly on the amount of Schwann cell outgrowth. The peripheral stump, reinnervation of which had been prevented, was compared with the tip of the central stump, where Wallerian degeneration occurs substantially as it does in the peripheral stump, but reinnervation quickly supervenes. When explanted 2, 3 or 4 days after severing the nerve, the two kinds of stump did not differ from each other in amount of Schwann cell outgrowth. But with longer periods of initial degeneration the outgrowth from the peripheral stump far exceeded that from the central stump.

We tentatively ascribed the striking difference of behaviour *in vitro* between central and peripheral stumps to the invasion of the central stumps by regenerating nerve fibres, which were absent from the peripheral stumps. Wallerian degeneration, we suggested, induces in Schwann cells a potentiality to migrate in appropriate conditions (such as tissue culture), but nerve fibres diminish this potentiality. Our hypothesis could not, however, be considered well-founded so long as it rested only on a comparison of central and peripheral stumps, which may differ in important respects other than the presence or absence of nerve fibres. Since the control of cell migration is of great general interest, we have now investigated the problem further by using peripheral stumps only, and comparing the amount of outgrowth in tissue culture from explants of stumps in which reinnervation had been prevented with those in which it had been allowed to occur.

## MATERIAL AND METHOD

Our material was taken from the peroneal division, and to a lesser extent the tibial division, of the sciatic nerve of rabbits. In one leg of each rabbit a reinnervated degenerated nerve was prepared, usually by crushing the whole sciatic nerve with smooth-tipped forceps at the level of the great trochanter, under nembutal and ether anaesthesia. At the same time the sciatic nerve on the other side was prepared as a non-reinnervated degenerated nerve, by crushing in the same way and then transecting the nerve a few mm. above the place crushed. Subsequent reinnervation of this nerve was prevented by suturing the central stump to the under-surface of the skin when the period of degeneration was to be 100 days; and in the shorter experiments by removing 1 cm. of the central stump. In a few experiments the sciatic nerve of both sides was crushed, but at different times, so as to provide comparable material of different periods of reinnervation.

The effect of crushing a nerve in this way has been investigated by Gutmann, Guttman, Medawar & Young (1942). Wallerian degeneration of the nerve fibres

occurs in the peripheral stump. Regenerating axons cross the site of injury after an average of 5.2 days had elapsed. When they enter the peripheral stump they travel rapidly down it, the leading axon tips averaging 4.4 mm. a day.

In a few experiments the reinnervated nerve was prepared by first severing the peroneal branch with scissors and then, after an interval, at a second operation, severing the tibial branch and suturing it by plasma clot to the peroneal peripheral stump (method of Young & Medawar 1940). Gutmann *et al* (1942) found that after suture of this kind, new axons passed the suture line an average of 7.3 days later, and the leading axon tips then travelled down the peripheral stump at an average rate of 3.5 mm./day. Control non-reinnervated nerves were prepared in these experiments by severing them with scissors.

The rabbits were mostly killed either about 25 days or about 100 days after the initial operation. About 8 cm. of each sciatic nerve was then removed, extending from the original operation site to a position well down the shank. Corresponding levels of the two sciatic nerves, reinnervated and non-reinnervated, from each rabbit were used for tissue culture or were fixed, usually a large series of different levels being prepared from each rabbit. Successively more distal levels of each reinnervated nerve will have undergone successively longer initial periods of degeneration before they were invaded by axons, varying in the crushed nerves from 5 days at the site of the crush to 23 days at a level 8 cm. beyond it (these periods are average figures). Correspondingly the length of time that axons have been present at the time of autopsy diminishes along the nerve.

Tissue culture was by hanging-drop technique, using fowl plasma and 8- to 9-day chick embryo extract. Explants were approximately 1 mm. lengths of the whole thickness of the peroneal nerves, half thickness of tibials (tibial explants are thereby made approximately the same size as peroneal explants). The epineurium was removed before culture. Usually a reinnervated and a non-reinnervated explant from the same level were placed together in each clot. After 3 days *in vitro* at 38° C the cultures were fixed and stained. The nuclei of outwandered Schwann cells, and sometimes of macrophages and fibroblasts (which in our classification included all cells neither Schwann cells nor macrophages), were then counted under the microscope.

We must briefly discuss the validity of the counting technique. Such differential counting, it must be emphasized, is a method of estimation, with an error like any other method. The error is no objection provided it is random and sufficiently small for statistically valid results to be practicable. We have attempted to reduce bias to a minimum by adhering to fixed criteria of classification. Scope for the exercise of bias will then chiefly come from the allocation of cells which do not clearly fall to one of the classes. Such 'indeterminate' cells certainly exist in our cultures, but they are relatively few, and bias in assigning them is negligible in accounting for our results. The validity of our interpretations also depends on the correlation of the classification of *in vitro* cell types with that determined by the usual histological methods. This has often been discussed; in the case of Schwann cells by Ingebrigtsen (1916), Murray, Stout & Bradley (1940) and others; and we need not pursue it further.

Mitoses and cell degenerations are much too uncommon in the outgrowth (and most of those that occur concern macrophages) for differences in their frequency to play a significant part in explaining differences in the population of the outgrowth at fixation. We have not considered any allowance for them necessary.

Statistical treatment, after transforming the variates to logarithms, was by analysis of variance, due allowance being made for disproportionate numbers in the subclasses. Treatment variance was tested against interaction variance.

## RESULTS

### (1) Schwann cells in tissue culture

We will consider first the numbers of Schwann cells that appear in the zones of outwandering after 3 days *in vitro*.

#### (1a) Existence of an inhibition

Table 1 shows the results obtained in the group of rabbits in which the nerve was interrupted about 100 days before culturing (hereafter called 'the 100-day group'). Each figure in the table is the mean number of Schwann cells of usually four to six explants from one region of one nerve. Each row gives the data for a single rabbit (designated A to G). Successive regions distal to the original site of crushing are given from left to right in the columns. Each column is double, showing the figure for the reinnervated nerve on the left, and (when available) for the corresponding region of non-reinnervated nerve on the right. It should be noted that the figures in the last column are not directly comparable with those of the other columns, since in this region (between 6 and 8 cm. below the site of crushing) we are dealing only with the anterior tibial branch of the peroneal, which provides explants only about half the size of those from the peroneal at higher levels. Table 2 gives the data, similarly arranged, for the group of rabbits operated by crushing the nerve about 25 days before culturing (hereafter called 'the 25-day group').

TABLE 1. 100-DAY GROUP

*Notes.* Mean numbers of Schwann cells appearing in tissue cultures. Each double column represents a region of the nerve, and is headed by its position in cm. below the site of crushing, and the approximate number of days new nerve fibres have been present in the reinnervated nerve (from data of Gutmann *et al.* 1942). On the left in each column (headed R) are figures for reinnervated nerves; on the right (headed non-R) for non-reinnervated nerves. Each row represents a single rabbit.

rabbit	0-0.5 cm. 94-95 days		0.5-2 cm. 90-94 days		2-4 cm. 86-90 days		4-5.5 cm. 82-86 days		6-8 cm. 77-81 days	
	R	non-R	R	non-R	R	non-R	R	non-R	R	non-R
A	3	82	21	76	37	183	59	70	32	92
B	3	39	7	84	9	58	8	113	11	78
C	6	15	7	91	—	—	6	200	37	219
D	9	35	9	90	—	—	2	26	100	229
E	16	75	5	76	8	81	19	167	—	—
F	—	—	11	88	5	66	—	—	—	—
G	0	—	2	—	3	—	—	—	9	—

TABLE 2. 25-DAY GROUP

rabbit	0-0.5 cm. 19-20 days		0.5-2 cm. 15-19 days		2-4 cm. 11-15 days		4-5.5 cm. 7-11 days		6-8 cm. 2-6 days	
	R	non-R	R	non-R	R	non-R	R	non-R	R	non-R
H	10	627	31	245	304	357	592	790	1075	230
I	2	127	34	152	55	415	159	129	580	273
J	1	65	36	147	—	—	495	99	37	38
K	114	655	162	423	—	—	2150	1620	379	350
L	17	309	76	540	114	390	—	—	—	—
M	0	—	24	—	149	—	351	—	554	—
N	4	—	39	—	89	—	—	—	590	—

Conventions as in table 1.

Comparing in table 1 the corresponding figures for reinnervated and non-reinnervated nerves it is clear that the number of Schwann cells growing out during culture is in the 100-day group profoundly depressed by the presence of new axons. We have most complete data for the region of nerve between 0.5 and 2 cm. below the site of crushing, and here the Schwann cells of the reinnervated nerves have mostly about 10 % of the activity of the non-reinnervated. The difference between the reinnervated and non-reinnervated nerves is significant at the 0.1 % level of probability in this region. The statistical analysis excludes rabbit G, which has no control nerve. Regenerated nerve fibres have been present in this region at least 90 days. In all other regions the effect of reinnervation is also in evidence, and is significant at the 5 % level of probability. Indeed, in all but one experiment the mean activity of the reinnervated explants is less than half that of the corresponding non-reinnervated explants. The visible effect of reinnervation is actually even more striking than these figures suggest, because from the non-reinnervated explants there usually project numerous fibrils of Schwann cell cytoplasm, devoid of nuclei, while these are largely absent from the reinnervated explants. This difference is illustrated in figures 1 and 2, plate 19.

The 25-day group, in table 2, likewise shows a considerably smaller outwandering of Schwann cells from the reinnervated nerves, in the regions down to 2 cm. below the site of crushing. In the most thoroughly investigated region, 0.5 to 2 cm., the reinnervated nerves average about 20 % of the non-reinnervated nerves in amount of outgrowth, and the difference between them is significant at the 1 % level of probability. The statistical analysis excludes rabbits M and N, which have no control nerves. Regenerated nerve fibres have been present for only about 15 to 20 days. The depression of outwandering by reinnervation is also significant, at the 1 % level of probability, in the half-centimetre immediately distal to the site of crushing. Beyond 2 cm. from the site of crushing the groups of reinnervated and non-reinnervated nerves at each region do not differ significantly at the 5 % level of probability. But we may suspect that in the 2 to 4 cm. region at least two rabbits (I and L) are showing the effects of the reinnervation which has existed here for 11 to 15 days. Only beyond 4 cm., where nerve fibres have been present less than 11 days, has the reinnervation as yet had no depressing influence on outwandering.

The experiments so far referred to concern reinnervated nerves prepared by crushing. In addition, we have six experiments in which the reinnervated nerve was prepared by suturing, as described in the section on method. The following results refer to the region between 0.5 and 2 cm. below the suture line:

(a) In four of these experiments the nerve was allowed to degenerate for 50 days after cutting before suture was performed. In one of these four, cultured 17 days after suture, the reinnervated nerve and its non-reinnervated control did not differ in outwandering; in two of them, cultured 37 and 47 days after suture, the Schwann cell outwandering of the reinnervated nerves averaged 36 and 29 % of that of the non-reinnervated controls: and in one, cultured 160 days after suture, the outwandering of the reinnervated nerve averaged 18 % of that of the control.

(b) In the other two of the six experiments with reinnervation by suture, the time between cutting and suture was only 8 days. In one of them, cultured 37 days after suture, the reinnervated nerve outwandering was 11 % of that of its control, which, however, was not from the exactly corresponding region; while in the other, cultured 110 days after suture, the outwandering of the reinnervated nerve was 21 % of that of its non-reinnervated control. These results indicate that the depression of Schwann cell outwandering produced by reinnervation after suture is comparable to that produced by reinnervation after crushing. Possibly outwandering is less diminished after suture than after crushing, but that is to be expected, since reinnervation is slower and less complete in many respects after completely transecting a nerve than after crushing it (Gutmann 1942; Gutmann & Guttmann 1942; Gutmann & Sanders 1943).

#### (1b) *Development of inhibition*

The 100-day group shows an inhibition of outwandering at all levels studied. In the 25-day group no inhibition occurs below 4 cm. from the lesion. In three supplementary experiments in which the reinnervated nerve was crushed 15 days before culture, there is no trace of inhibition below 2 cm. Evidently inhibition spreads peripherally in the course of reinnervation. It travels down the nerve well behind the fastest moving of the axon tips, which according to Gutmann *et al.* (1942) would probably be about 9 cm. below the lesion in the 25-day group and 4.5 cm. in the 15-day nerves. The fact that inhibition travels down the nerve behind the fastest moving of the axon tips means, of course, that at any point there is a period after the arrival of the first axons before detectable inhibition starts.

A striking feature of our results is the profound inhibition of outwandering which has already developed in the 25-day group. The activity of the reinnervated explants of the 0.5 to 2 cm. region averages approximately 20 % of that of the non-reinnervated explants, and this percentage is quite a reliable estimate (its 0.95 fiducial limits are approximately 10 and 30 %). There is therefore a very rapid initial development of inhibition in this region of the reinnervated nerves, especially if we take into account the period which elapses between operation and the arrival of the first axons, together with that between the latter and the onset of inhibition. If the number of Schwann cells free to migrate continued to diminish at this initial

rate, outwandering would be zero in a few more days. Since it is not nearly zero 75 days later, in the 100-day group, we must consider whether the advance of inhibition is merely much slower beyond 25 days after crushing the nerve, or whether inhibition has at 25 days already reached its maximum.

In the 100-day group of experiments the outwandering from reinnervated explants of the 0.5 to 2 cm. region is certainly far less (average, weighted according to the different number of explants from each rabbit, 12 nuclei) than in the 25-day group (weighted average 70 nuclei). But, as was to be expected from the results of Abercrombie & Johnson (1942), the non-reinnervated nerves also show a considerably smaller outwandering in the 100-day group (weighted average 84 nuclei) than in the 25-day group (weighted average 340 nuclei). The outwandering from reinnervated nerves must therefore be treated for purposes of comparison as a proportion of that from corresponding non-reinnervated nerves; and while this proportion is actually a little larger in the 25-day than in the 100-day group, it is not significantly different by *t* test between the two groups.

We have two other experiments which concern this problem. In them the sciatic nerve of one side was crushed, and cultured 300 days later together with corresponding regions of the unoperated nerve of the other side. In a nerve crushed as long ago as this the fibre spectrum near the site of crushing has returned practically to normal (Gutmann & Sanders 1943). The 0.5 to 2 cm. region of these nerves has an average outwandering of 2.4 nuclei per explant. This exceeds, but does not differ significantly from, the outwandering of the unoperated control nerves. But it does differ significantly, at the 1 % level, from the outwandering of the 100-day group of reinnervated nerves (average 12 nuclei per explant); and the difference is perhaps greater than would be expected from the known decline in outwandering from non-reinnervated nerves (Abercrombie & Johnson 1942), though our data are certainly not decisive on this point. We must therefore leave it that after 25 days the observed changes in outwandering of reinnervated nerves *can* be explained by the known decline of outwandering from non-reinnervated nerves, together with sampling error; though we may suspect that there is a small further depression of outwandering by continued reinnervation.

In two regions the course of development of inhibition seems to be a little different from that in the 0.5 to 2 cm. region already discussed. In the half-centimetre immediately below the site of crushing, which is likely to have been traumatized by the operation, inhibition is unexpectedly severe in the 25-day group. Outwandering of the reinnervated nerves as a proportion of the non-reinnervated is significantly smaller (at the 5 % level of significance, by *t* test) than in the 0.5 to 2 cm. region. The latter region has been reinnervated for an average of only 3 days less than the traumatized region. Except for rabbit K in table 2 the absolute amount of outwandering produced by this region is practically the same in the 25-day group as in the 100-day group.

In contrast to the traumatized region, the anterior tibial branch of the peroneal nerve 6 to 8 cm. down in the shank shows throughout the period studied an unexpectedly small inhibition. In the two 300-day reinnervated nerves the outwandering from the nerve of the shank region (averages 40 nuclei per explant) far



exceeds that from the 0.5 to 2 cm. region (average 2.4 nuclei) and that from the corresponding shank region of the unoperated control nerves (2.3 nuclei). In the 100-day group of experiments (table 1) there seems also to be a disparity between the outwandering of reinnervated nerves in the 6 to 8 cm. region and that in other regions; a disparity both in absolute amount of outwandering (considering that the nerve is considerably smaller in the shank than in the other regions) and in amount relative to the outwandering of corresponding non-reinnervated nerves. The differences in the 100-day group are by themselves of doubtful statistical significance; but taken in conjunction with the results from the 300-day nerves, they suggest that the shank region is much less affected by reinnervation than are more proximal regions.

In summary, the development of inhibition of outwandering in the best known region, 0.5 to 2 cm. below the lesion, probably proceeds as follows: after the invasion by the first nerve fibres there are a few days (perhaps about 5 to 10) when no inhibition is detectable; then come a few days (perhaps about 10) of rapidly deepening inhibition; and finally a relatively stable period of no change or only slightly increasing inhibition. In other regions of the nerve these same phases probably succeed each other, though there is evidence that they differ in duration and intensity.

#### (1c) *Initial stimulation*

In earlier work Abercrombie & Johnson (1942) found evidence that, in the earliest stages of degeneration, outwandering from the tip of the central stump was greater than that from the tip of the corresponding peripheral stump. This suggested that the effect of reinnervation on Schwann cells might initially be a stimulation of their outwandering activity. A similar effect is indeed suggested in some of these experiments where the outwandering from the reinnervated nerves exceeds that from the non-reinnervated (for instance in table 2, rabbits H and I in the 6 to 8 cm. region and rabbit J in the 4 to 5.5 cm. region). In other experiments, however, there is no sign of it. In particular, in one experiment in which the nerve was crushed 15 days before suture, there is a good range of explants from the traumatized region along 5 cm. of crushed and of non-reinnervated control nerves; yet no trace of stimulation was apparent. The existence of the stimulation must therefore be considered in doubt, and if it is a reality it must be very transient.

#### (2) *Other cells in tissue culture*

We have so far considered only the inhibition of migration of Schwann cells by reinnervation. Macrophages and fibroblasts also appear in the zone of outwandering. Are they also inhibited by reinnervation? Table 3 gives the results of counting these cell types in ten experiments (four from the 25-day group, six from the 100-day group, used in the previous analysis of Schwann cell activity). Each experiment consists of corresponding cultures of the degenerated and reinnervated nerves from the same rabbit; only the level from 0.5 to 2 cm. below the site of crushing has been used. The numbers of macrophages and fibroblasts are considerably more variable (from rabbit to rabbit and from explant to explant) than the

numbers of Schwann cells; but the variation is entirely random, and no tendency whatever to inhibition of fibroblasts and macrophages by reinnervation is discernible. The inhibition is therefore specific to Schwann cells.

TABLE 3

*Note.* Mean numbers of fibroblasts and macrophages appearing in tissue cultures of region of nerve 0.5–2 cm. below the site of crushing. Other conventions as in table 1.

100-day group					25-day group				
rabbit	fibroblasts		macrophages		rabbit	fibroblasts		macrophages	
	R	non-R	R	non-R		R	non-R	R	non-R
A	464	202	224	65	H	12	24	10	49
B	107	98	92	108	I	28	30	60	113
C	86	147	45	124	J	42	8	207	86
D	29	25	29	22	L	2	2	122	83
E	2	43	35	93					
F	4	6	12	27					

Although we are confident that our methods of differential counting are adequate, it is worth recording that proof of a depression of outwandering by reinnervation does not depend on separate enumeration of Schwann cells. In the 100-day group the reinnervated nerves have an outwandering which at the 5 % level of probability is significantly less than that of the non-reinnervated nerves when all kinds of cells are included in the counts. Taking Schwann cells and fibroblasts together, or Schwann cells and macrophages together, reinnervated nerves have significantly less outwandering than non-reinnervated at the 1 % level. The result of combining Schwann cells and macrophages is interesting because of the suggestion made by Weiss & Wang (1945) that the majority of macrophages found in the zone of outwandering of peripheral nerve cultures are derived by conversion of Schwann cells which have already migrated out. The effect of pooling the different cell types in the 25-day group is to obscure the differences between reinnervated and non-reinnervated nerves, except for combined Schwann cell and fibroblast counts, which are significantly (at the 5 % level) depressed in reinnervated nerves.

### (3) *Cell population in vivo*

It is known that during the first 25 days of degeneration of the rabbit sciatic nerve there is a rapid increase of cell population, mainly of Schwann cells (Abercrombie & Johnson 1946), and this increase largely but not entirely accounts for the increase in outwandering *in vitro* which occurs during the same period. An obvious way in which the depression of outwandering by reinnervation might be caused is through a depression of the Schwann cell population. To test this hypothesis we investigated the cell populations in pairs of peroneal nerves, one reinnervated and one non-reinnervated from each rabbit. Transverse and longitudinal sections were prepared from pieces of the nerve fixed in Bouin or Susa, the nerves of the two sides of each rabbit being treated identically. The region examined was between 0.5 and 1 cm. below the site of crushing, in four rabbits of the 100-day

group. Axons must have been present in this region for all but the first 6 or 7 days after the operation. The number of nuclei in a complete transverse section of  $7\mu$  thickness for these four pairs of nerves was as follows, the reinnervated being given first, the non-reinnervated second: 1763 and 1585; 1602 and 1612; 1985 and 2417; 1436 and 1187. No correction of these figures is necessary for size of nuclei (see Abercrombie & Johnson 1946), since measurements of nuclear lengths in longitudinal section showed no significant differences between reinnervated and non-reinnervated nerves. It is sufficiently evident from these four rabbits that the depression of outwandering is not due to an effect of reinnervation on the number of Schwann cells present in the nerve. Indeed, it appears improbable that reinnervation has any effect whatever on cell population. The two nerves reinnervated for 300 days also had populations entirely within the normal range of non-reinnervated nerves of this period of degeneration (2109 and 1723; the unoperated peroneal nerves from the other side of these rabbits contained 805 and 509 nuclei respectively).

#### DISCUSSION

We have established that the amount of Schwann cell migration in tissue culture from explants of those degenerated nerves which have been reinnervated for more than a certain time is less than that from explants of corresponding non-reinnervated degenerated nerves. In considering possible explanations of this phenomenon two conclusions are particularly relevant: (a) the effect is specific to Schwann cells, and this is so even though macrophages are also present in the Schwann tubes of a degenerating nerve, and (b) the effect occurs very strongly after only a short period of reinnervation, when the new nerve fibres are very small, and consequently very unlikely to be blocking the tubes mechanically (see Gutmann & Sanders (1943), for data on fibre size in crushed nerves; Holmes & Young (1942) in sutured nerves).

Many workers, such as Nageotte (1922), Speidel (1935), Weiss (1939) and Young (1942), have remarked on the affinity of Schwann cells and nerve fibres, which is manifested in growth and regeneration by a tendency to spread over each other in peculiarly intimate contact. Nerve fibres and Schwann cells, indeed, form close contacts with a variety of surfaces (Weiss 1945), but their mutual relationship seems to be an especially close one. The obvious hypothesis in the circumstances is that Schwann cells become immobilized by prolonged contact with nerve fibres; and the action of such contact is best explained, we suggest, by the development of a strong specific adhesion between the two elements. This is in accordance with the theoretical views of Weiss (1947).

The hypothesis we suggest therefore is that outwandering in tissue culture is depressed when such adhesion is well developed in the nerve. It is very strongly developed in a normal intact nerve. In the peripheral stump of a divided nerve we may suppose that the rapid rise in amount of outwandering with time of degeneration (Abercrombie & Johnson 1942) is at least partly due to release of Schwann cells from their adherence to nerve fibres, because of the destruction of the latter. The invasion of such a stump by new axons re-establishes a considerable degree of adhesion, depressing outwandering again. The tip of the central stump of a divided

nerve also undergoes the degenerative process, and the first stages of release from adhesion of the Schwann cells thereby occur. Re-inhibition sets in again after a few days, however, as new nerve fibres sprout from the stumps of the old.

It will be important to correlate the onset of inhibition of outwandering in a reinnervated peripheral stump with other processes of reinnervation. The axon tips immediately stick to and spread on Schwann cells when they touch them, but this apparently produces no inhibition. There is a possible correlation of the inhibition with the beginning of myelination, which Speidel (1932) has shown to involve close collaboration between Schwann cell and axon. During regeneration a front of active myelination proceeds down the nerve well behind the axon tips, with time relations, as far as they are known (see Rexed & Swensson 1941; Young 1942), probably compatible with those of the depression of migration. The onset of the inhibition of outwandering does not seem to be related to any 'maturation' of the Schwann cells during Wallerian degeneration. Our results, especially those with sutured nerves, strongly suggest that the migration of Schwann cells can be depressed at any stage in Wallerian degeneration.

Once the inhibition begins, it develops very rapidly in the proximal part of the peripheral stump, reaching in less than 20 days an intensity such that outwandering is merely about one-fifth of that from a non-reinnervated nerve. Thereafter it deepens only very slowly, if at all. It is however perhaps significant that outwandering was practically that of a normal nerve in the proximal part of the nerves which we cultured after a reinnervation of 300 days, the fibre spectrum of which should also have returned to practically normal (Gutmann & Sanders 1943).

Migratory activity *in vitro* probably reflects migratory activity at least in one situation *in vivo*: at the freshly cut tip of a stump (Abercrombie & Johnson 1942; Young 1942). In this situation the inhibition produced by reinnervation has been noticed by Nageotte & Guyon (1916), Young (1942) and Rexed (1942). Young's opinion was in fact that 'it seems that Schwann cells do not continue their migratory activities when they are reinnervated'. It is generally agreed that this migration of Schwann cells from the cut surfaces of a nerve, by bridging the gap between the stumps, is important in producing adequate recovery after surgical suture (see Young 1942), and our results should have some bearing on this topic. Union of the stumps is experimentally better after slightly delayed than after immediate or long delayed suture (Holmes & Young 1942). The curve of Schwann cell migratoriness *in vitro* during degeneration (Abercrombie & Johnson 1942) has been invoked as part of the explanation of these results, but to be adequate this explanation must be supplemented by the results of the present paper. These results indicate that the outwandering which occurs during a short period immediately following suture will be of especial importance, since the arrival of reinnervating nerve fibres soon depresses further outwandering. Primary suture, and to a lesser extent long-delayed suture, should for this reason be at a disadvantage compared with suture performed near the time of maximum outwandering. It is reasonable to suppose that the experimental work explains, at least in part, the disadvantage of primary suture actually found in surgical practice (Zachary & Holmes 1946; Spurling & Woodhall 1946).

The interpretation of the inhibition of Schwann cell migratory activity by adhesion of Schwann cells to nerve fibres recalls analogous embryological phenomena. Holtfreter's beautiful experiments (1939, 1947) have demonstrated selective adhesiveness and non-adhesiveness between different types of cells in early amphibian embryos. Weiss (1941, 1945) and Schmitt (1941) have contributed much to the interpretation of these phenomena. Such adhesions have been invoked in explanation of the extension of one cell surface over another. The present work has emphasized a different aspect of the phenomenon, discussed by Weiss (1947). Although it may encourage mutual extension of contact, and thereby cell movement, specific adhesion may also be of great importance in *stopping* cellular migration where migration involves the parting of adherent surfaces; and conversely loss of adhesion may release such cellular migration. In embryonic development orderly cellular migration is of course very frequent, and such migration may start and stop through the operation of cell adhesions. In the adult it may be that the general immobility of the majority of cells (excluding the wandering cells) is partly due to specific surface adhesions; and breakdown of the latter may therefore be an important process in the onset of migration in adult tissues. Such an onset of migration occurs very generally where active proliferation starts, as in wound-healing and in the development of malignant tumours.

#### SUMMARY

1. The amount of outwandering in tissue culture from explants of reinnervated and non-reinnervated degenerated rabbit sciatic nerves has been compared, by counting the number of cells which come to lie outside each explant after 3 days *in vitro*.

2. Using nerves interrupted 25 days before culturing, the amount of Schwann cell outwandering from explants taken from the proximal part of peripheral stumps is much less in the reinnervated than in the corresponding non-reinnervated nerves. The distal regions of the stumps do not differ.

3. When the nerves had been interrupted 100 days before culturing, this difference in amount of Schwann cell outwandering between reinnervated and non-reinnervated nerves is present to about the same degree as in those interrupted 25 days before: but the effect is now found along the whole length of the peripheral stump.

4. The outwandering of cells other than Schwann cells is not affected by reinnervation; neither is the total cell population within the nerve.

5. These results are interpreted in terms of a specific adhesion supposed to develop between Schwann cells and nerve fibres, which prevents the migration during tissue culture of a proportion of the Schwann cells.

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FIGURE 1. Reinnervated nerve.



FIGURE 2. Non-reinnervated nerve.

Representative regions of the zones of outwandering from explants, after 3 days in tissue culture, of the two sciatic nerves from one rabbit. Both nerves interrupted 100 days before culturing; one crushed, permitting reinnervation, the other completely severed, preventing reinnervation. Formalin fixed, haematoxylin stained. Linear magnification  $\times 260$ .





# Metabolic activity in tissue transplants.

## Hormone-induced formation of fructose and citric acid in transplants from accessory glands of reproduction

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[Plates 20 to 22]

A combined chemical and cytological study of the behaviour of transplants from certain accessory glands of reproduction in the rat was carried out.

It was found that subcutaneous transplants of coagulating gland and seminal vesicle were capable of producing considerable amounts of fructose and citric acid in total anatomical separation from the male reproductive system.

In transplants containing coagulating gland and seminal vesicle tissue both fructose and citric acid were formed. In those from coagulating gland alone only fructose was produced, but citric acid was absent. In this respect the metabolic behaviour of the grafts was identical with that of the intact organs.

Following castration, coagulating gland transplants lost their ability to form fructose. This was fully restored by treatment with testosterone propionate. Upon cessation of the hormone treatment the process of fructose formation in the transplants was again brought to a standstill.

Grafts of coagulating gland could be successfully grown in female rats and brought to a state of fructose secretion by subjecting the female hosts to injections of male sex hormone.

The post-castrate retrogressive changes as well as the hormone-induced recovery symptoms were studied in the transplants parallel with similar changes in the intact glands *in situ*. The chemical findings were corroborated by the histological examination.

### 1. INTRODUCTION

During the passage through the male genital tract the spermatozoa receive the secretions of several accessory organs of reproduction—vasa deferentia, seminal vesicles, prostate gland, Cowper's gland and other urethral glands. These secretions constitute together the so-called seminal plasma which forms the natural environment and nutrient medium for the sperm cells. The development of the accessory glands depends on the presence of the male sex hormone. As a result of gonadectomy they regress rapidly but promptly resume their normal appearance and function in response to treatment with male sex hormone (Deanesly & Parkes 1933; Korenchevsky, Dennison & Brovsin 1936; Moore 1935, 1937; Moore & Gallagher 1929; Price 1936, 1947; Zuckerman & Parkes, 1938). Some of the accessory glands lend themselves well to subcutaneous transplantation, and in the rat, for instance, grafts weighing several hundred milligrams can be grown within a few weeks from a few milligrams of subcutaneously implanted donor tissue; the growth and cellular differentiation of the transplanted tissue entirely depend upon the action of the testicular hormone, in complete analogy with the accessory glands (Price 1941, 1942).

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Hitherto the physiological conditions prevailing in the transplanted tissues were assessed mainly on the basis of cytological studies. However, recent biochemical researches revealed the secretion by the accessory glands of two substances, fructose and citric acid. In the course of the studies on the distribution of fructose and citric acid in the accessory glands of various animal species (Davies & Mann 1947; Humphrey & Mann 1948; Mann 1946, 1948; Mann, Davies & Humphrey 1948; Mann & Parsons 1947) it was found that in the rat fructose is secreted by the coagulating gland (anterior prostate) and dorsal prostate, and citric acid by the seminal vesicle proper and ventral prostate (Humphrey & Mann 1948). It was also found that the processes which lead to the formation of these two metabolites are strictly controlled by the male sex hormone (Mann & Parsons 1947; Mann, Davies & Humphrey 1948). In view of the close similarity in behaviour towards the male hormone, of accessory glands and the grafts grown from them, the present study was undertaken to examine the chemical activity of the grafts, and their ability to produce fructose and citric acid, in complete anatomical separation from the rest of the genital apparatus. It will be seen below that when small portions of immature and functionally undeveloped rat coagulating gland and seminal vesicle were dissected from young donors and transplanted under the skin of hosts, male or female rats, the grafts grew readily in response to testicular hormone, and when fully developed showed a high content of fructose and citric acid. The ability to form fructose was restricted to grafts from coagulating gland, whereas those grown from the combined tissues of coagulating gland and seminal vesicle produced both fructose and citric acid (preliminary communication, Mann, Lutwak-Mann & Price 1948).

## 2. EXPERIMENTAL MATERIAL AND METHODS

### *Transplantation technique*

Grafting of the tissues followed the technique developed by Price (1941, 1942) for the cytological study of rat prostate and seminal vesicle. Freshly killed young rats, aged 15 to 50 days, served as donors; with the aid of a binocular dissecting microscope small fragments of tissue were dissected either from the combined seminal vesicle plus coagulating gland or from the latter alone, and at once inserted in subcutaneous sites on the abdomen of the host. The animals chosen as hosts, male and female, varied in age from 30 to 115 days; some were normal rats, others were gonadectomized at various stages of the experimental periods; of these some were left untreated whereas others received testosterone propionate, 200  $\mu$ g. daily by subcutaneous injections. The majority of the experimental animals were Sprague-Dawley albino rats, but some were from the inbred stock maintained at the Department of Zoology, University of Chicago. Altogether about 100 male rats were used as donors, and thirty male and female rats served as hosts. As a routine six pieces of donor tissue weighing between 1 and 3 mg. each were transplanted into a single host in two rows of three, one row on each side of the abdomen. Subcutaneous transplants were allowed to grow for 16 to 96 days. At autopsy grafts were found attached to the skin or abdominal wall from which they could easily be dissected. The weights of the grafts varied considerably according to the period

of growth and the treatment of the hosts; grafts nearly 1 g. in weight were found in hosts subjected to prolonged testosterone propionate treatment. Transplant material in quantities suitable for chemical analysis was deproteinized with ice-cold trichloroacetic acid, and the extracts were later used for the estimation of fructose and citric acid. Simultaneously, small samples were fixed in Bouin's fluid and later sectioned serially for histological examination. When grafts were grown in male hosts, the accessory glands of the host were also analyzed both chemically and histologically. In order to obtain sufficient quantities of graft tissue for chemical analysis from castrated male hosts, in which the growth of grafts of accessory gland tissue is very limited, the tissue was transplanted into normal hosts and allowed to grow for a period of time. When it could be determined by palpation that the grafts were well established and large, the host was castrated. After a period of regression the host was killed and the grafts and the host accessory glands were analyzed.

#### *Chemical methods*

Estimations of fructose and citric acid were carried out in protein-free trichloroacetic acid extracts. Fructose determinations were carried out as described by Mann (1946, 1948), citric acid by the method of Speck, Moulder & Evans (1946). It proved instructive to express quantitative results both as mg./100 g. fresh tissue (mg. %) as well as per weight of organ. The glycolysis of the accessory glands and the graft tissue was measured manometrically in presence of added glucose. The procedure was that employed by Mann & Lutwak-Mann (1948) for the study of the carbohydrate metabolism in rat seminal vesicle; the production of acid was estimated by measuring the  $\text{CO}_2$  output using Ringer-bicarbonate and a gas mixture of 95 %  $\text{N}_2$  and 5 %  $\text{CO}_2$ . Results were expressed in terms of  $Q_{\text{CO}_2}$  ( $\mu\text{l. CO}_2$  produced in 1 hr. at 37° C by 1 mg. tissue, dry weight).

#### *Histological methods*

The serially cut sections of grafts and accessory reproductive organs were stained in haematoxylin. The details concerning the cytology of male accessory organs in the rat, and the methods used for handling and staining the histological sections were those described by Moore, Price & Gallagher (1930) for the rat prostate, and by Moore, Hughes & Gallagher (1930) for the seminal vesicle; in these papers the coagulating gland is referred to as anterior prostate, in accordance with the nomenclature of Engle (1926). Photomicrographs showing the structure of the host and graft coagulating gland are given at the end of the paper (plates 20 to 22); in each instance a low-power view ( $\times 70$ ) is shown with high magnification ( $\times 700$ ).

### 3. FRUCTOSE AND CITRIC ACID IN ACCESSORY ORGANS IN RELATION TO AGE

In this investigation it was essential to use for transplantation material obtained at an age when the donor's accessory organs had either none, or only very little, of fructose and citric acid. Studies concerning the relationship between age and fructose and citric acid content in accessory glands were carried out before on rabbits (Davies & Mann 1947) and bull-calves (Mann, Davies & Humphrey 1949).

With regard to rats, three groups of animals were used, (1) eight animals aged 40 days, (2) five animals 85 days old, and (3) two 1-year-old rats; in each instance the dissected organs of a given group were pooled and thus represent average values. The results are collected in table 1, which provides separate data for ventral prostate, dorsal prostate, coagulating gland and seminal vesicle.

TABLE 1. FRUCTOSE AND CITRIC ACID CONTENT OF RAT ACCESSORY ORGANS  
IN RELATION TO AGE

Average data obtained by analysis of (1) eight animals, 40 days old; (2) five animals, 85 days old; (3) two animals, 350 days old.

	ventral prostate			dorsal prostate			coagulating glands			seminal vesicles		
age of rat (days)	40	85	350	40	85	350	40	85	350	40	85	350
weight of organ (mg.)	70	320	780	47	229	438	10	150	221	31	727	1568
fructose ( $\mu$ g./organ)	0	0	0	10	212	134	4	248	293	—	—	25
citric acid ( $\mu$ g./organ)	31	124	198	—	41	55	0	0	0	10	313	462

It is obvious that there is no chance of introducing any appreciable quantities of fructose or citric acid with graft material from 40-day-old donors, especially as the weight at implantation did not exceed a few milligrams. The values for the fructose and citric acid content in the three age groups differ strikingly when compared as absolute quantities per animal; in terms of concentration, i.e. as mg./100 g. tissue, they indicate that even at the early age of 40 days, shortly before the onset of spermatogenesis, the male accessory organs of the rat, like those of rabbit and bull, are already active. For example, at the age of 40 days, the coagulating glands contained only 4  $\mu$ g. fructose per animal, but this small quantity corresponded to a concentration of 40 mg. %. At the age of 85 days there was 248  $\mu$ g. fructose in the coagulating gland per animal, corresponding to a concentration of 166 mg. %. The seminal vesicles, on the other hand, which form citric acid but not fructose, can be seen to contain at the age of 40 days only 10  $\mu$ g. citric acid, but at 85 days as much as 113  $\mu$ g. citric acid; in terms of concentration, however, the corresponding figures were 33 mg. % at 40 days, and 44 mg. % at 85 days.

#### 4. GROWTH AND METABOLIC ACTIVITY IN TRANSPLANTED TISSUES. FORMATION OF FRUCTOSE AND CITRIC ACID IN RESPONSE TO VARIATIONS IN DONOR TISSUE, GONADECTOMY, TESTICULAR HORMONE AND SEX OF HOST

##### *Transplants from combined tissues of coagulating gland and seminal vesicle*

The analysis of grafts grown in normal male hosts from donor material containing the seminal vesicle proper plus coagulating gland showed that both citric acid and fructose were produced in the transplants. After 3 months' subcutaneous development the transplanted tissue reached a considerable weight, and chemically it differed little from the host tissues (table 2). Manometric experiments showed that such transplants readily glycolyzed added glucose;  $Q_{CO_2}$  for transplants was 5.3, for host seminal vesicle 2.3, and for host coagulating gland 4.6.

TABLE 2. FRUCTOSE AND CITRIC ACID IN TRANSPLANTS FROM COMBINED TISSUES OF SEMINAL VESICLE AND COAGULATING GLAND

Rats male, I and II, implanted at the age of 50 days with 15 days old donor tissue, autopsied 96 days later.

rat	...	weight of tissue (seminal vesicle plus coagulating gland) (mg.)		fructose (mg. %)		citric acid (mg. %)	
		I	II	I	II	I	II
organs of host		1210	1329	68	49	58	36
transplant tissue		1450	1400	43	24	17	60

*Transplants from coagulating gland alone*

That the metabolic processes in transplanted tissues correspond closely to those in the intact organs themselves can best be judged from experimental results obtained with grafts from coagulating gland alone. These had no citric acid at all, whereas their fructose concentration was sometimes equal to that of the host (table 3).

TABLE 3. FRUCTOSE IN TRANSPLANTS FROM COAGULATING GLAND

no.	male host		age of donor tissue (days)	fructose	
	age at implantation (days)	age at autopsy (days)		coagulating gland of host (mg. %)	graft from coagulating gland (mg. %)
1	40	67	40	140	147
2	70	107	50	138	168
3	150	177	47	200	44

In order to be able to draw an even closer comparison between the functional capacity of the grafts and that of the host coagulating glands themselves, a histological examination was carried out parallel to the chemical analyses.

The histological and cytological character of a 40-day-old coagulating gland is shown in figures 9 and 10, plate 21. This is the age at which the gland is producing 40 mg. % fructose. The epithelium of such a gland varies from cuboidal to moderately tall columnar, and some vacuolization of the cytoplasm can be seen. The structure of a normally developed coagulating gland in a mature 67-day-old rat (rat no. 1 of table 3) is illustrated by figures 1 and 2, plate 20. Figure 1 gives a low-power view of the gland showing several typical acini with multiple villous projections into the lumina, whereas figure 2 gives the fine cytological details of the tall columnar epithelium as it appears under the high-power magnification, revealing clearly the vacuolated character of the cell protoplasm, and the location, half-way between the basal membrane and the lumen end, of large nuclei which stain in a characteristically heavy homogeneous fashion. A direct comparison between the structure of the coagulating gland of the host, and that of a graft from the coagulating gland, is possible through a study of figures 1 and 2 and figures 3 and 4, plate 20. The entire field of the low-magnification figure 4 can be seen to be occupied by a single, tremendously distended acinus. A small portion of this acinus (from the upper

right corner portion of the low-power field) is shown under high magnification in figure 3. The epithelium of the graft as shown by this photomicrograph is in many parts much lower than that in figure 3, but the higher epithelium resembles very closely the epithelium of the normal coagulating gland itself, both in the structure of the cytoplasm as well as in details relating to the location and staining character of the cell nuclei. The low 'stretched'-appearing epithelium is apparently due to the accumulation of large amounts of secretion in the graft acini.

#### *Effect of castration and testicular hormone*

In view of the previously established influence of testicular hormone on the formation of fructose and citric acid in accessory glands, a series of experiments was designed to examine the corresponding processes in grafted tissues.

When an animal was used as host at prepubertal age, that is, before there is any significant fructose formation, and autopsied at an age of 2 months, it was found that the host coagulating gland contained 140 mg. % and the graft 147 mg. % of fructose (table 3). However, when rats were castrated at the age of 113 to 125 days, that is, at a time when they are fully mature, the result was a considerable fall of fructose content not only in the host glands but in the grafted tissue as well (table 4, rats 3, 4, 5, 6 and 7). It would appear at first glance that fructose in the host organs was more affected by the removal of gonads than in the grafts. However, it must be borne in mind that, unlike the intact organ, a graft has no outlet, and that once-accumulated fructose cannot be voided. In addition, it is highly probable that the graft tissue has little ability to metabolize fructose, since it has been shown previously that, unlike spermatozoa, the seminal vesicle and coagulating gland utilize fructose only very poorly (Mann & Lutwak-Mann 1948).

TABLE 4. EFFECT OF CASTRATION AND TESTOSTERONE ON FRUCTOSE CONTENT OF TRANSPLANTS FROM COAGULATING GLAND

host no.	donor days of age	male host, days of age at				host's coagulating glands		grafts from coagulating glands	
		implan- tation	castra- tion	testosterone treatment	autopsy	weight (mg.)	fructose (mg. %)	weight (mg.)	fructose (mg. %)
1	37	37	37	37-92	92	200	155	170	130
2	37	37	37	37-92	92	360	224	180	144
3	40	50	125	— <sup>a</sup>	146	54	8	862	6
4	56	56	113	—	144	31	3	104	
5	56	56	113	—	144	51	3	223	34
6	51	51	118	—	139	53	3	265	
7	51	51	118	—	139	45	3	318	34
8	28	75	75	75-132	154	74	5	766	12

In rats which lost the ability to produce fructose as a result of castration a striking change was brought about by treatment with testosterone propionate. For instance, rats 1 and 2 in table 4 were castrated at the early age of 37 days and implanted at the same time with donor tissue from litter-mates. From that day onwards they received daily injections of 200  $\mu$ g. testosterone propionate for 55 days until they

were 92 days old. On autopsy both the host coagulating gland and the grafts showed a remarkably high fructose content.

The dependence of fructose formation upon the stimulus provided by the testicular hormone was best demonstrated in the experiment in table 4 in which rat 8 was castrated and received graft material when 75 days old. For another 57 days it received daily injections of 200  $\mu$ g. testosterone propionate, a dose more than adequate to stimulate maximal fructose production. Then the injections were discontinued for 3 weeks. At autopsy this rat showed only 5 mg. % fructose in its coagulating gland and 12 mg. % in the grafts. It was interesting to note that the weight of the graft tissue did not decline nearly as rapidly as did the content of fructose.

In order to gain a better understanding of the nature of the post-castrate changes in grafts, a histological study was made on portions of the same material as used for chemical analysis.

Figures 5 and 6, plate 21, illustrate the effect of castration on the histological appearance of the coagulating gland, figures 7 and 8, plate 21, the post-castrate changes in the coagulating gland transplant of the same rat. This animal has been referred to before as no. 3 in table 4. It was implanted with coagulating gland material when 50 days old, and castrated at 125 days of age, by which time the subcutaneous grafts had reached a considerable size. Three weeks later it was killed and the grafts as well as the coagulating glands themselves analyzed both chemically and histologically. The host's coagulating gland can be seen to have undergone a considerable involution as a result of the castration which was performed 3 weeks earlier. The acini were small and surrounded by large amounts of interacinous connective tissue (figure 5). The epithelium appeared very low, and the nuclei inside the epithelial cells were crowded together, giving a stratified appearance to the epithelium (figure 6). Unlike the acini in the host's coagulating gland, those in the subcutaneous graft were large and distended with secretion (figure 8); this behaviour of the grafts no doubt explains their weight (see table 4). However, in spite of the considerable size the graft acini did not present secretory activity. Their epithelium was very low and stretched, and the nuclei in the epithelial cells were small (figure 7).

The histological appearance of the tissues in the castrated male rat is in striking contrast to the behaviour of the analogous tissues in a castrated but testosterone propionate-treated animal (rat 1, table 4). In the coagulating gland of the hormone-treated castrate the acini were not greatly distended, but they showed many villous projections which distinguish the normally secreting gland, and there was abundant secretion (figure 11, plate 21). Similarly, the epithelium of these acini (figure 12, plate 22) bore all those features which are characteristic of the normal non-castrated animal. If anything, it appeared higher than in the non-castrated animal, probably because the daily dose of 200  $\mu$ g. testosterone propionate as used in the treatment of the castrated animal was in excess of the amount of hormone required by a castrate for the restoration of the secretory cells to their normal activity. The grafts of the castrated and hormone-treated rat showed the same high degree of activity as the coagulating glands of the host itself. They were, however, much more distended than the gland proper owing to the accumulation of secretion

(figure 14, plate 22). Their epithelium was stretched and flat in some places, no doubt as a result of pressure by the secretory fluid, but in others it looked normal both with regard to cytoplasm and the nuclei (figure 13).

*Transplants of coagulating gland in female hosts*

The behaviour of grafts of male accessory glands in the female body furnished yet another convincing proof of the close relationship between the function of testicular hormone and the metabolic activity of coagulating gland tissue. Following the routine described above, a series of experiments was carried out in which spayed and non-spayed female rats were used as hosts. There was no difficulty in maintaining the growth and development of male tissue under continuous treatment with testosterone propionate. The results can be seen from table 5; the analysis of graft material showed as much as 100 to 200 mg. % fructose in the transplants, after a period of 3 to 8 weeks' testosterone propionate administration. There was no evidence in these experiments that intact ovaries interfered with the graft development, as similar results were achieved in normal and in spayed rats.

TABLE 5. FRUCTOSE IN COAGULATING GLAND TRANSPLANTS IN FEMALE RATS

host no.	donor days of age	female host, days of age at				graft tissue	
		implan- tation	spaying	testosterone treatment	autopsy	weight (mg.)	fructose (mg. %)
1	37	71	—	71- 92	92	310	112
2	37	71	—	71- 92	92	275	191
3	28	77	77	77-132	132	800	102
4	22	77	77	77-132	155	790	12
5	22	77	77	77-132	155	898	3

In conformity with the experience gained with male hosts, the cessation of hormonal treatment caused a sharp fall in the fructose-forming activity of the grafts in female hosts; no more than 12 and 3 mg. % fructose, respectively, was found in two female rats in which testosterone propionate treatment was discontinued (table 5, rats 4 and 5).

## DISCUSSION

The technique of animal tissue transplantation and the study of the developing grafts is one of the most fascinating subjects in biology and has already yielded valuable information concerning cellular differentiation and the nature of the various factors which govern the development of animal tissues. Transplants of prostate and seminal vesicles have been used by several workers as indicators of the presence of male hormone or as a means of studying the reactions of these glands in response to introduced hormones (Price 1941). Hitherto the interpretation of the physiological conditions prevailing in transplanted tissues was largely based upon histological examination. The study of certain biochemical aspects concerning the metabolic activity of the graft tissue as compared with the intact organs is therefore something of a departure from the older lines of thought.



Some time ago fructose was established as a normal constituent of seminal plasma in bull, ram, boar, man, rabbit and several other species, and its origin traced to certain accessory glands of reproduction (Mann 1946). This opened up the possibility of an investigation into the conditions under which fructose is produced. In due course it was found that its appearance in the semen and in the accessory glands is strictly governed by the male sex hormone (Mann & Parsons 1947). At this stage it was of considerable biological interest to inquire to what extent the ability to form fructose was inherent in the accessory glands themselves, and how far it was bound up with the remainder of the involved neural and hormonal apparatus of the male generative tract. Transplantation technique seemed to offer the best chance of solving these problems, as it makes feasible the development of accessory glands in complete anatomical separation from the genital tract; in addition, previous extensive cytological studies have demonstrated that in suitable hosts such grafts grow, differentiate and exhibit a normal functional histological state.

The choice of the rat as the experimental animal was guided by several considerations. Details of transplantation were largely worked out for this species by Price (1941, 1942). In addition, from earlier work it was known that in the rat, unlike in other animals, the seminal vesicle proper, while producing citric acid, contains practically no fructose (Humphrey & Mann 1948). This made it possible to follow the fate of these two metabolites independently of each other as well as to establish the degree of metabolic specificity.

It is clear from the experimental results that in the transplants one possesses a very handy and sensitive tool for the biochemical study of the action of hormones and other agents, in this case the male sex hormone, upon metabolic processes. By introducing variations in the type of donor tissue, it was also possible to demonstrate that these fragments of tissue, even from very young donors, are capable of developing into grafts endowed with precisely the same metabolic ability as the gland from which they originate, and that the particular processes under investigation are subject in the graft to the influence of the testicular hormone in exactly the same manner as in the host organs.

For the time being the actual mechanism whereby the testicular hormone stimulates the formation of fructose and citric acid in the accessory glands remains obscure. But the experiments in which active grafts of male tissue developed in female hosts, provided these were adequately supplied with testosterone, indicate clearly the primary importance of the hormonal agent, from the cellular and chemical point alike, as against relatively less essential factors such as the sex of host or the anatomical and neural link with the genital tract.

Moore, Price & Gallagher (1930) have carried out cytological studies on the post-castrate behaviour of the coagulating gland in rat. They found that in distinction to other accessory organs the retrogressive changes in the coagulating gland are not readily perceptible histologically until about the tenth day, and become well pronounced only at about 20 days after castration when it is possible to diagnose clearly such symptoms as lowering of epithelium, decrease in the size of nuclei, disappearance of the basal membrane and a reduction in the size of Golgi

bodies. The cytological picture of the grafts from the coagulating gland in the present study resembled closely the findings made previously for the parent organ itself. It is satisfactory to note that on the whole the chemical response to castration and testicular hormone, both in the graft and in the host gland, as judged by the level of fructose, was matched by the microscopical appearance of the tissues.

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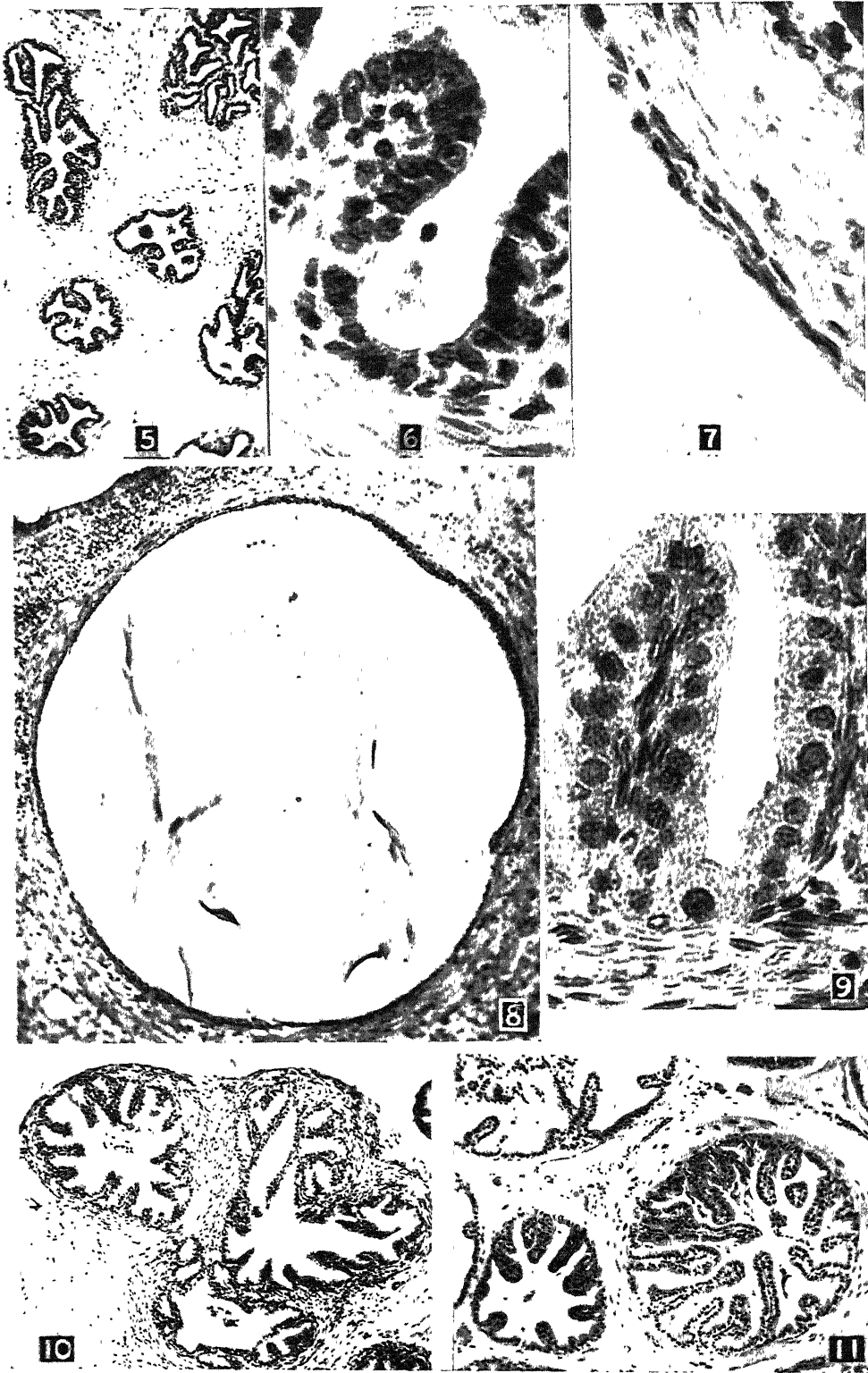
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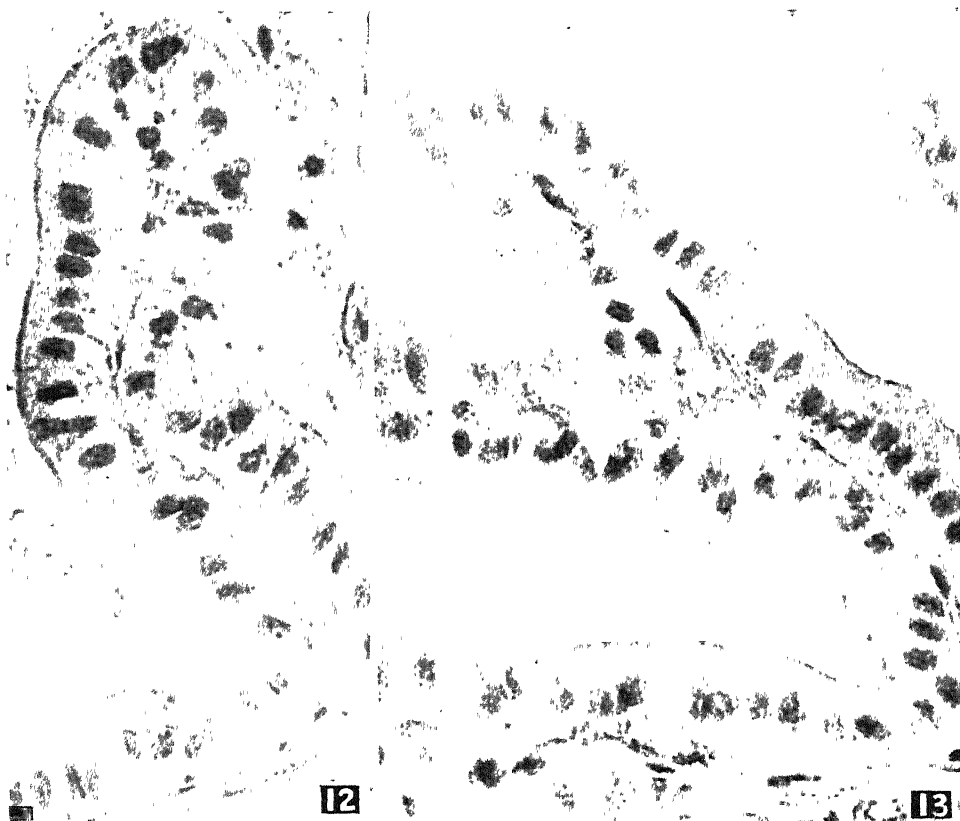
#### DESCRIPTION OF PLATES 20 TO 22

##### PLATE 20

- FIGURE 1. Coagulating gland of a 67-day-old male rat, host to the graft shown in figures 3 and 4. (Magn.  $\times 70$ .)  
 FIGURE 2. A part of figure 1 showing the histological character of the epithelium. (Magn.  $\times 700$ .)  
 FIGURE 3. A part of figure 4 showing the normal character of some of the epithelium of the graft. Compare with figure 2. (Magn.  $\times 700$ .)  
 FIGURE 4. Coagulating gland graft from a normal male host (graft period 27 days). Compare with the host coagulating gland in figure 1. Note the greatly distended acinus, the accumulated secretion and the generally flat epithelium due to the accumulation of secretion. Some of the high epithelium is shown in figure 3. (Magn.  $\times 70$ .)









## PLATE 21

FIGURE 5. Coagulating gland of a 146-day-old castrated male rat (castrated for 21 days), host to the graft shown in figures 7 and 8. (Magn.  $\times 70$ .)

FIGURE 6. A part of figure 5 showing the low epithelium that is typical of a castrated rat. (Magn.  $\times 700$ .)

FIGURE 7. A part of figure 8 showing the very low epithelium of the graft. Compare with figure 6. (Magn.  $\times 700$ .)

FIGURE 8. Coagulating gland graft from a castrated male host (total graft period 96 days; the male host was normal for the first 75 days and castrated for the last 21 days). Compare with the host coagulating gland in figure 5. Note the distention of the acinus, the accumulated secretion remaining in the graft and the low epithelium. (Magn.  $\times 70$ .)

FIGURE 9. A part of figure 10 showing moderately high columnar epithelium. (Magn.  $\times 700$ .)

FIGURE 10. Coagulating gland of a 40-day-old male rat. (Magn.  $\times 70$ .)

FIGURE 11. Coagulating gland of a 92-day-old castrated male rat (castrated for 55 days and injected with 200  $\mu$ g. of testosterone propionate daily for 55 days), host to the graft shown in figures 13 and 14. Compare with the coagulating gland of the normal rat in figure 1 and the castrated rat in figure 5. (Magn.  $\times 70$ .)

## PLATE 22

FIGURE 12. A part of figure 11 showing the normal character of the epithelium of the host coagulating gland. Compare with figures 2 and 6. (Magn.  $\times 700$ .)

FIGURE 13. A part of figure 14 showing the normal character of some of the epithelium of the coagulating gland graft. Compare with figure 12. (Magn.  $\times 700$ .)

FIGURE 14. Coagulating gland graft from the host shown in figures 11 and 12. Note that the field contains only a part of one greatly distended acinus and that the epithelial height varied from low to high. Compare with the host coagulating gland in figure 11. (Magn.  $\times 70$ .)

# The antigenic relationship of some mammalian spermatozoa

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Antisera prepared in goats, sheep and rabbits against the spermatozoa of rodents, cross-agglutinated strongly spermatozoa of other rodents, weakly those of ferret and dog, and not at all those of bull, goat and fowl.

After absorption of these antisera with whole spermatozoa of the species against which they had been prepared, homologous reactions were abolished, but heterologous reactions persisted. When absorbed with heterologous spermatozoa of any species the various antisera failed to agglutinate spermatozoa of that species only.

Spermatozoa which had been fragmented by grinding absorbed homo- and hetero-agglutinins from the homologous antiserum, but only reduced the agglutinin content of heterologous antisera.

The results suggested that antigens on the surface of spermatozoa of rodents of one species were more deeply situated in those of other species, and that the spermatozoa of rabbit and guinea-pig were more closely related to one another than they were to those of mouse.

## INTRODUCTION

Antisera prepared against spermatozoa display a high degree of organ specificity (Landsteiner & van der Scheer 1927), and fail to react with serum of the same species (Henle 1938). They react strongly with spermatozoa of the species used for immunization; but cross-reactions, usually much weaker, occur with spermatozoa of different but related species and even of different orders (Mudd & Mudd 1929). Thus a strong antigenic resemblance has been found between the spermatozoa of bull, sheep and deer, and weaker but distinct cross-reactions between the spermatozoa of bull and man (Henle 1938).

Henle and his colleagues (Henle, Henle & Chambers 1938) found they could separate the heads and tails of mammalian spermatozoa with the magneto-constriction oscillator, and used whole spermatozoa or their component parts for preparing, absorbing and testing antisera. By complement fixation and agglutination tests they demonstrated in bull spermatozoa three species-specific antigens, and by means of complement-fixation tests alone, three different cross-reacting antigens, one in the spermatozoal tails, one on the surface of their heads, and one detectable only in the heads of broken cells.

Employing agglutination tests with absorbed anti-spermatozoal sera, Snell (1944) detected in the spermatozoa of one strain of mice antigens which were lacking in the spermatozoa of another strain. For demonstrating differences between the serum proteins and red blood corpuscles of closely related species, Landsteiner & van der Scheer (1924) found that complement fixation and precipitin tests were less satisfactory than agglutination tests. An attempt has therefore been made to study, by means of agglutination tests, the antigenic relationship of mammalian spermatozoa of several different species.



## MATERIAL AND METHODS

### *Preparation of anti-spermatozoal sera*

Full details of the production of most of the anti-spermatozoal sera have been given previously (Smith 1949). In addition, a female goat (G.6) was treated with fifteen weekly intraperitoneal injections of goat spermatozoa, and 10 days after the last injection 200 ml. of its blood was withdrawn and the serum separated. The rabbits MR. 4, 5 and 6 received six weekly intravenous injections followed after an interval of 3 months by five weekly subcutaneous injections and three intraperitoneal injections of guinea-pig spermatozoa. The rabbits R. 14, 15 and 16 were given six weekly intravenous injections of guinea-pig spermatozoa, and were bled out 18 days after the last injection.

### *The slide agglutination test*

The method for performing and recording the results of slide agglutination tests has been described previously (Smith 1949).

### *Absorption of sera*

#### *With whole unwashed spermatozoa*

A dense suspension of spermatozoa was prepared in 4 ml. of 0.85 % NaCl using the contents of the vasa deferentia and epididymides of two adult guinea-pigs, or of ten adult rats, or of fifty adult mice. In the case of rabbit spermatozoa, 1 ml. of freshly collected semen was diluted with an equal quantity of 0.85 % NaCl. The spermatozoal suspension was centrifuged at about 2000 r.p.m. for 30 min., to separate the diluted epididymal fluid or seminal plasma. The deposit of spermatozoa was resuspended in 1.5 ml. of 0.85 % NaCl, and an equal volume of a 1 in 10 dilution of antiserum was mixed with it. After standing for 1 hr. at room temperature, a drop was examined microscopically to ensure that there was a large excess of spermatozoa not agglutinated. The tube was left overnight in the cold room at 2° C, and next day centrifuged for 30 min. at about 2000 r.p.m. The supernatant fluid, consisting of absorbed serum corresponding to a 1 in 20 dilution of the original serum, was removed and its titre for spermatozoal agglutinins determined.

#### *With washed spermatozoa*

The deposit of spermatozoa from a suspension of epididymal spermatozoa, or from rabbit semen, obtained as above, was resuspended in 10 ml. of 0.85 % NaCl and centrifuged at about 2000 r.p.m. for 30 min. The supernatant fluid was discarded, and the spermatozoa washed a second time in the same way. The deposit of washed spermatozoa was suspended in 1.5 ml. of 0.85 % NaCl, and used to absorb 1.5 ml. of a 1 in 10 dilution of antiserum, as before.

#### *With ground spermatozoa*

A suspension of washed spermatozoa in 1.5 ml. of 0.85 % NaCl, prepared in the manner described, was transferred to a mortar containing approximately 7.5 g. of dry washed sand. It was ground with a pestle for 30 min. or until no intact heads or tails of spermatozoa were recognizable under the microscope. The sand containing

the fragments and soluble material from the spermatozoa was transferred to a centrifuge tube and used to absorb 1.5 ml. of the 1 in 10 dilution of antiserum. In a control tube the same quantity of serum was treated with 7.5 g. of dry washed sand, and 1.5 ml. of 0.85% NaCl.

#### *Fragments of ground spermatozoa*

The procedure for grinding spermatozoa was repeated. 1.5 ml. of 0.85% NaCl was added to the sand and fragments and stirred for 10 min. to extract readily soluble material. The mixture was then centrifuged at approximately 2000 r.p.m. for 30 min., or until on microscopical examination the supernatant fluid was free from particles. After removing the supernatant fluid, 1.5 ml. of the diluted serum was mixed with the sand and spermatozoal fragments and left to absorb as before.

#### *Extract of ground spermatozoa*

The supernatant fluid from the above, generally 1.2 to 1.4 ml. in volume, was used to absorb an equal quantity of diluted antiserum.

## RESULTS

### *Unabsorbed sera*

All the anti-spermatozoal sera were tested against spermatozoa from rabbit semen, and from the epididymides of guinea-pigs and mice; some were, in addition, tested against spermatozoa from dog, bull, goat and fowl semen, and from the epididymides of ferrets. Table 1 summarizes the results, and shows that most of the antisera gave a range of cross-reactions, the titre of each serum being highest in the homologous reaction. The sera G.2 to 5 from goats, and S.1 from a sheep, immunized with the spermatozoa of laboratory rodents, gave strong cross-reactions with the spermatozoa of rodents of other species, weaker reactions with those of ferret and dog, and none with those of bull or fowl. They agglutinated goat spermatozoa to the same titre as did normal goat serum. The serum from the goat (G.6), after a prolonged course of injections of goat spermatozoa, failed to agglutinate the spermatozoa of rodents, and its titre for goat spermatozoa was no higher than that of serum from the same animal before immunization was started. The sera from rabbits injected with rabbit spermatozoa failed to agglutinate the spermatozoa of rabbits and guinea-pigs. With the exception of M.R. 4, none of the antisera prepared in rabbits against guinea-pig spermatozoa had a significant titre for rabbit spermatozoa; some of them had high titres for mouse spermatozoa.

### *Sera absorbed with whole, unwashed spermatozoa*

Because of their high titres in homologous and heterologous reactions, the goat sera G.2 to 5 were used for these experiments.

Each serum was absorbed with the homologous spermatozoa, and subsequently tested against spermatozoa of that and of several other species. The results are given in table 2, and show that in every case the homologous reaction was abolished,

TABLE 1. AGGLUTINATION TITRES OF SERA AGAINST SPERMATOZOA OF DIFFERENT SPECIES

serum no.	animal immunized	spermatozoa used for immunizing	spermatozoa used for testing							
			rabbit	guinea-pig	mouse	ferret	dog	bull	goat	fowl
G.0	goat	none	<10	<20	<20	—	20	20	80	40
G.2	goat	rabbit	1280	640	320	320	80	10	80	40
G.3	goat	rat	640	640	640	160	20	20	80	40
G.4	goat	mouse	640	640	1280	320	40	20	80	40
G.5	goat	guinea-pig	320	640	640	320	80	20	80	40
G.6	goat	goat	<10	<10	<20	—	0	—	80	40
S.1	sheep	rat	160	160	640	—	10	0	—	40
R.0	rabbit	none	0	0	20	—	—	0	—	40
R.8	rabbit	guinea-pig	0	640	320	80	80	0	—	40
R.10	rabbit	guinea-pig	0	160	320	20	—	0	—	0
R.12	rabbit	guinea-pig	0	80	40	—	—	0	—	20
R.14	rabbit	guinea-pig	0	160	80	40	—	—	—	—
R.15	rabbit	guinea-pig	0	160	80	40	—	—	—	—
R.16	rabbit	guinea-pig	0	160	80	—	—	—	—	—
MR.4	rabbit	guinea-pig	80	160	20	—	20	—	—	—
MR.5	rabbit	guinea-pig	0	160	80	—	20	—	—	—
MR.6	rabbit	guinea-pig	0	160	40	—	20	—	—	—
R.1 to 6	rabbit	rabbit	0	0	—	—	—	—	—	—
P.1 to 6	rabbit	rabbit	0	0	—	—	—	—	—	—

Note. The figures in heavy type represent the titres for homologous reactions.

but that, contrary to expectation and to the results of Henle *et al.* (1938) and of Landsteiner & van der Scheer (1924), the heterologous reactions persisted, often with high titres.

TABLE 2. THE EFFECT OF ABSORPTION WITH HOMOLOGOUS SPERMATOZOA ON AGGLUTINATION TITRES

serum no.	homologous spermatozoa	treatment	spermatozoa used for testing		
			rabbit	guinea-pig	mouse
G. 2	rabbit	unabsorbed	1280	640	320
		absorbed	0	80	160
G. 3	rat	unabsorbed	640	640	640
		absorbed	80	40	640
G. 4	mouse	unabsorbed	640	640	1280
		absorbed	160	160	0
G. 5	guinea-pig	unabsorbed	320	640	640
		absorbed	40	0	320

After absorption with heterologous spermatozoa the various sera failed to agglutinate spermatozoa of the species used for absorption, but continued to agglutinate to different titres those of other species. Table 3 shows the results. When rabbit spermatozoa were the absorbing agents, titres for guinea-pig spermatozoa were reduced, and vice versa; in both cases high titres remained for mouse spermatozoa. Absorption with mouse spermatozoa left high titres for rabbit and guinea-pig spermatozoa. Rat spermatozoa were less effective in absorbing agglutinins for the spermatozoa of mouse than for those of rabbit and guinea-pig.

TABLE 3. THE EFFECT OF ABSORPTION WITH HETEROLOGOUS SPERMATOZOA ON AGGLUTINATION TITRES

serum no.	spermatozoa used for absorption	spermatozoa used for testing		
		rabbit	guinea-pig	mouse
G. 2	none	1280	640	320
	guinea-pig	40	0	160
	mouse	160	160	0
	rat	40	40	40
G. 3	none	640	640	640
	rabbit	0	160	320
	guinea-pig	40	0	320
	mouse	80	160	0
G. 4	none	640	640	1280
	rabbit	0	80	1280
	guinea-pig	40	0	640
	rat	40	40	320
G. 5	none	320	640	640
	rabbit	0	80	320
	mouse	80	320	0
	rat	40	80	160

These results suggested that immunization with the spermatozoa of any one species had led to the production of a number of agglutinins, some of which were more active against and more easily absorbed by spermatozoa of other species.

*Sera absorbed with spermatozoa treated in different ways*

The absorbing power of washed and unwashed spermatozoa was compared in case soluble substances from the seminal plasma or epididymal fluid combined with agglutinating antibodies and subsequently parted with them in presence of spermatozoa of another species, or in any other way interfered with the absorbing power of the cells. As shown in tables 4 and 5, spermatozoa were, in most cases, slightly better absorbing agents after washing than before.

Absorption of the antisera G. 2 and G. 4 with ground homologous cells abolished heterologous reactions, whereas absorption with ground heterologous spermatozoa did not completely abolish the homologous reaction. Washing the spermatozoa before grinding them did not improve their absorbing power (tables 4 and 5). Fragmented guinea-pig spermatozoa were more efficient than those of mouse in removing agglutinins for rabbit spermatozoa from their homologous antiserum G. 2. Absorption with ground-up rabbit spermatozoa completely removed agglutinins for guinea-pig spermatozoa from the anti-mouse spermatozoal serum G. 4, and *vice versa*; in neither case was the titre for mouse spermatozoa much reduced. The antigens brought into action by grinding were associated with the fragments of the spermatozoa, fluid extracts of the various preparations of ground spermatozoa having little absorbing power.

These results suggested that the spermatozoa of rabbit, guinea-pig, and mouse possessed a number of common antigens, and that antigens superficially placed on the tails of spermatozoa of one species were present within the tails of spermatozoa of other species. While such deeply placed antigens were capable of provoking antibody formation after parenteral injection of whole cells, they were incapable of absorbing antibodies *in vitro* until the cells had been disintegrated by grinding. It appeared as though the spermatozoa of rabbit and guinea-pig were, antigenetically, more closely related to one another than they were to those of mouse.

#### DISCUSSION

For production of antisera against the spermatozoa of rodents, goats proved superior to rabbits. They responded better to immunization, yielding sera with higher agglutinin titres in homologous and heterologous reactions, and information unobtainable from the anti-spermatozoal sera prepared in rabbits. Thus goats were readily immunized against the spermatozoa of both rabbit and guinea-pig by injection of either, whereas the serum of rabbits immunized against the spermatozoa of guinea-pig usually failed to agglutinate those of rabbit, and serum from rabbits chronically injected with rabbit spermatozoa agglutinated neither. Using the goat sera, and depending on agglutination tests, a strong antigenic resemblance was found between the spermatozoa of rabbit, guinea-pig, rat, and mouse, and a weaker relationship of the spermatozoa of these rodents to those of ferret and dog, and none to those of bull, goat, or fowl. By absorbing the antisera with fragmented spermatozoa as well as with washed and unwashed whole spermatozoa, a wider distribution of antigens was demonstrated than would have been assumed from straight agglutination tests depending only on surface antigens.

TABLE 4. THE EFFECT OF ABSORBING WITH SPERMATOOZOA TREATED IN DIFFERENT WAYS ON THE AGGLUTINATION TITRES OF THE ANTI-RABBIT SPERMATOZOAL SERUM G.2

spermatozoa used for absorption		spermatozoa used for testing		
species	treatment	rabbit	guinea-pig	mouse
none	—	1280	640	320
rabbit	unwashed	0	160	320
	washed	0	80	80
	unwashed and ground	0	0	0
	washed and ground	0	0	0
	fragments	0	0	0
	extract	640	640	80
guinea-pig	unwashed	160	0	160
	washed	80	0	80
	unwashed and ground	40	0	0
	washed and ground	40	0	0
	fragments	80	0	0
	extract	640	160	80
mouse	unwashed	640	320	0
	washed	320	160	0
	unwashed and ground	—	80	0
	washed and ground	80	40	0
	fragments	160	80	0
	extract	640	320	80
rat	unwashed	80	0	0
	washed	0	0	0
	unwashed and ground	0	0	0
	washed and ground	0	0	0
	extract	1280	320	160

TABLE 5. THE EFFECT OF ABSORBING WITH SPERMATOOZOA TREATED IN DIFFERENT WAYS ON THE AGGLUTINATION TITRES OF THE ANTI-MOUSE SPERMATOZOAL SERUM G.4

spermatozoa used for absorption		spermatozoa used for testing		
species	treatment	rabbit	guinea-pig	mouse
none	—	640	640	1280
rabbit	unwashed	0	80	1280
	washed	0	40	1280
	unwashed and ground	0	0	640
	washed and ground	0	0	640
	fragments	0	0	640
	extract	160	160	1280
guinea-pig	unwashed	40	0	1280
	washed	20	0	1280
	unwashed and ground	0	0	640
	washed and ground	0	0	320
	fragments	0	0	640
	extract	—	—	1280
mouse	unwashed	640	320	0
	washed	320	160	0
	unwashed and ground	0	0	0
	washed and ground	0	0	0
	fragments	0	0	0
	extract	320	160	—

Three points of particular interest, two technical and one theoretical, emerge from this work. In studying the antigenic relationships of spermatozoa of different species facts will be missed if deeply placed antigens are not considered as well as superficial ones. Fallacies may be introduced if the spermatozoa to be tested are not washed, because the seminal and epididymal fluids contain antigens similar to those present on the spermatozoa. Finally, the problem is raised whether the seminal and epididymal antigens are derived from the breakdown of spermatozoa, or whether they and the spermatozoal antigens have a common source in the testis.

I am much indebted to Dr A. A. Miles, National Institute for Medical Research, for advice and for reading the manuscript. My best thanks are due to Dr A. S. Parkes, F.R.S., for stimulating criticism and invaluable help.

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BAKERIAN LECTURE  
A region of biosynthesis

BY H. RAISTRICK, F.R.S.

*London School of Hygiene and Tropical Medicine*

(Delivered 12 May 1949—Received 31 May 1949)

[PLATES 23 TO 26]

INTRODUCTION

The particular region of biosynthesis about which I propose to speak is that part of the kingdom of living things occupied by the fungi, particularly those fungi popularly known as 'moulds', with occasional reference to some higher fungi and also to lichens, which are of course symbionts of algae and fungi. It is, I think, not inappropriate that this should form the subject of a Bakerian Lecture, since the founder of this lecture, Mr Henry Baker, F.R.S., who was an ardent microscopist, described in 1742 the spores of the fungus *Lycoperdon*,\* the common puff ball (1).† In the following year he gave a good description of *Pilobolus*, a mould of the mucor type, which he found growing on a culture of black mud from the river Thames (2).

The exact position of fungi in the scheme of living things is still doubtful, since, to quote Dr John Ramsbottom (3), 'if organisms must be either plants or animals, then fungi are plants with a nutrition resembling that of animals as they do not possess chlorophyll. If, however, chlorophyll is the hall mark of plant phylogeny the fact has to be faced that fungi probably never possessed it.' Because of the absence of chlorophyll, fungi can be cultivated only on media containing pre-formed organic matter, but this very fact makes moulds particularly suitable for biochemical investigation, since they grow well on very simple media.

In spite of this fact, real interest in the chemistry of moulds may be said to date from 1891 with Carl Wehmer's classical observations that when *Aspergillus niger* is grown on sugar solutions oxalic acid is formed in considerable quantities (4, 5, 6, 7), and that citric acid is a metabolic product of certain mould species to which he gave the generic name *Citromyces* (8, 9). For the next thirty years only an occasional paper appeared on this subject, and this was the position when I was fortunate enough to be able to begin systematic work on the subject in 1923 with a small team of enthusiastic colleagues in the research laboratories of Nobel's Explosives Company Ltd., Ardeer, Scotland. This work was carried on there until 1929 (10), since when it has been continued in the Department of Biochemistry at the London School of Hygiene and Tropical Medicine.

\* A list of species cited in the text with the authorities for each is printed in the appendix to the lecture.

† Because of the large number of literature references cited, the custom usually followed in this journal of quoting in the text the names of authors and year of publication has been altered. Each publication referred to is given a number and the same numbering is used in the list of references at the end of the lecture.

## EXPERIMENTAL METHODS

The general plan of the work was the investigation of the products of metabolism of pure cultures of species and strains of moulds when grown under controlled conditions on simple, reproducible and chemically defined culture media. We have used almost exclusively one or other of the two following media:

Czapek-Dox medium		Raulin-Thom medium	
glucose	50 g.	glucose	75 g.
NaNO <sub>3</sub>	2 g.	tartaric acid	4 g.
KH <sub>2</sub> PO <sub>4</sub>	1 g.	ammonium tartrate	4 g.
KCl	0.5 g.	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.6 g.
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5 g.	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.25 g.
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.01 g.	K <sub>2</sub> CO <sub>3</sub>	0.6 g.
distilled water	1 l.	MgCO <sub>3</sub>	0.4 g.
		FeSO <sub>4</sub> .7H <sub>2</sub> O	0.07 g.
		ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.07 g.
		distilled water	1.5 l.

It will be seen that in the Czapek-Dox medium the only source of organic matter is glucose, and in the Raulin-Thom medium glucose + tartaric acid. The other elements essential for growth, nitrogen, phosphorus, potassium, sulphur, magnesium and the trace elements are present as mineral salts. Hence, any of the organic metabolites which I shall describe must have arisen by synthesis from glucose or glucose and tartaric acid.

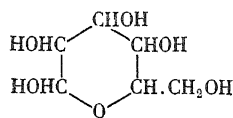
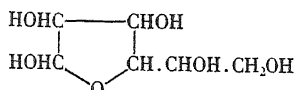
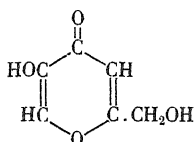
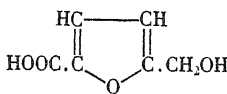
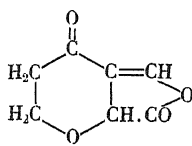
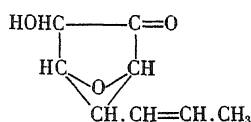
The medium, distributed in 350 ml. amounts in 1 l. conical flasks plugged with cotton-wool, is sterilized, inoculated with a pure culture of the species or strain of mould under investigation, and incubated in the dark at 24° C. Hence all the cultural conditions are standardized and reproducible, the only variant being the species or strain of mould used, so that I think it may be claimed fairly that structural differences in chemically related metabolic products may be attributed to differences in the enzyme systems present in different species or in strains of the same species.

The cultures are harvested when the residual glucose is about 0.5%. The mould mycelium is separated by filtration from the culture fluid without sterilization, washed with water, pressed and dried in a vacuum oven at 40° C. It is then ground to a fine powder, extracted with suitable solvents, and any extractives are purified by the usual chemical methods.

Any metabolites in the clear culture filtrate and mycelium washings are isolated by extraction with suitable solvents, or by the addition of suitable precipitants, or even by vacuum evaporation when they may occasionally crystallize out.

During the last twenty-six years a large number, certainly approaching two hundred, of mould metabolic products have been isolated in a state of purity by my colleagues and myself. It will not be possible to deal with all of them to-day, nor would I wish to weary you with a mere catalogue of fungal metabolic products. My purpose is rather to try to trace the interrelationships in chemical structure between the different types of mould metabolic products described by ourselves and other workers, and to suggest how some of the more complex ones may arise by bio-synthesis from the simpler ones.

## KOJIC ACID, PATULIN, 2-HYDROXYMETHYL-5-FURANE CARBOXYLIC ACID AND TERREIN

I  
glucopyranoseII  
glucofuranoseIII  
kojic acidIV  
2-hydroxymethyl-5-furane  
carboxylic acidV  
patulin (etc.)VI  
terrein

The first group of mould metabolic products is chosen because its members show some of the closest relationships occurring in mould metabolites to the glucose molecule, either in its pyranose (structure I) or furanose form (II). Their inter-relationships in structure are not particularly clear, though they all contain a bridged oxygen ring.

Kojic acid (III), originally isolated by Yabuta from culture filtrates of *Aspergillus oryzae* (11), has been described since then by many workers as a metabolite of a large number of different species of *Aspergillus* and is obtained occasionally in yields of over 50 %.

Patulin (V) was discovered independently and almost simultaneously during the last war in a number of laboratories in different countries. This is the reason why it now appears in the literature under a variety of names—clavacin from *A. clavatus* (12), clavatin from *A. clavatus* (13), claviformin from *Penicillium claviforme* (14, 15), expansin from *P. expansum* (16, 17) and patulin from *P. patulum* (18). Kojic acid and patulin may, I think, be legitimately regarded as derivatives of glucopyranose (I).

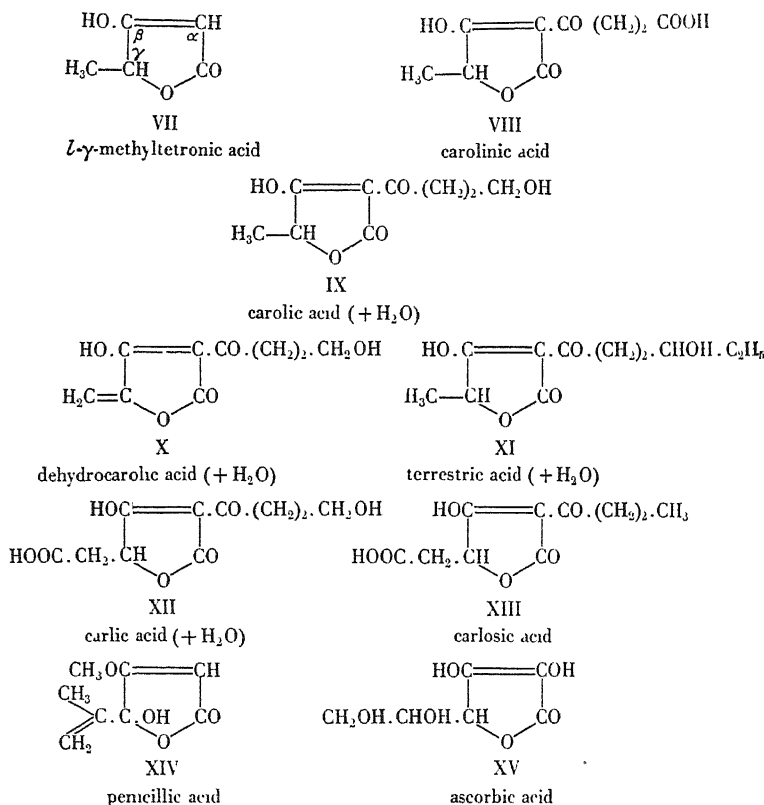
2-Hydroxymethyl-5-furane carboxylic acid (IV) obtained from *Aspergillus glaucus*, *A. clavatus*, *A. niger*, *A. oryzae* and *A. wentii* by Sumiki (19, 20), and terrein (VI) from *A. terreus* (21, 22) may be regarded, though perhaps with less certainty, as derivatives of glucofuranose (II).

## DERIVATIVES OF TETRONIC ACID

The next group of metabolic products is a series of nine derivatives of tetronic acid, all of which were isolated from the culture filtrates of species of *Penicillium* or *Aspergillus*. The acids from *Penicillium charlesii*, *l*- $\gamma$ -methyltetronic acid (VII), carolic acid (IX), carolinic acid (VIII), carlic acid (XII) and carlosic acid (XIII) were obtained in a total yield of about 14 % of the glucose metabolized (23). The

structural formulae for a number of these tetronic acids are given in their hydrated form for ease of comparison.

We regard  $\gamma$ -methyltetronic acid (VII) (24) as the parent substance of this group. Carolic acid (IX) (25) may be regarded as derived from it by substitution of the 4-carbon side-chain in the  $\alpha$  position. Carolinic acid (VIII) (25) differs from carolic acid only in having a carboxyl group in place of a carbinol group, and dehydrocarolic acid (X) from *P. cinerascens* (26) in having a  $\gamma$ -methylene group in place of the  $\gamma$ -methyl group. Terrestric acid (XI) from *P. terrestre* (27) is clearly an ethyl carolic acid. Carlic acid (XII) (28) has the same structure as carolic acid, with the exception that a carboxymethyl group takes the place of the  $\gamma$ -methyl group in carolic acid. Carlosic acid (XIII) (28) may be regarded as a reduced carlic acid having a terminal methyl group in place of  $\text{CH}_2\text{OH}$ . Carlosic acid was also obtained along with dehydrocarolic acid from *P. cinerascens* (26).



Penicillic acid (XIV) was described originally in 1913 by the American workers Alsberg & Black (29) who obtained it from *P. puberulum*. It was isolated from *P. cyclopium*, and its molecular structure was determined in my department (30). This structure has since been confirmed by an elegant synthesis carried out by Dr R. A. Raphael in 1948 (31) in Messrs May and Baker's research laboratories and at the Imperial College, London. The presence of a methoxy group will be noticed, a feature of common occurrence in mould metabolic products. Its structural relationship to  $\gamma$ -methyltetronic acid is obvious.

Ascorbic acid (XV) was identified along with much larger amounts of citric acid by Geiger-Huber & Galli in 1945 from cultures of *Aspergillus niger* (32). It is surely more than a coincidence that these two substances are also present together in the juice of citrus fruits.

TABLE 1. ACID HYDROLYSIS PRODUCTS OF TETRONIC ACIDS

acid	mols.	mols.	mols.
$\gamma$ -methyltetronic	1 CO <sub>2</sub>	1 acetoin	—
carolic	1 CO <sub>2</sub>	1 acetoin	1 butyrolactone
carlic	2 CO <sub>2</sub>	1 acetoin	1 butyrolactone
terrestrial	1 CO <sub>2</sub>	1 acetoin	1 <i>l</i> - <i>n</i> -hexanolactone (ethyl-butyrolactone)
carolinic	1 CO <sub>2</sub>	1 acetoin	1 succinic acid
carlosic	2 CO <sub>2</sub>	1 acetoin	<i>n</i> -butyric acid
dehydrocarolic	1 CO <sub>2</sub>	1 diacetyl	1 butyrolactone

An outstanding feature of most of the members of this group of substances is the ease with which the whole molecule splits apart on hydrolysis with boiling, dilute mineral acids. The hydrolysis products of seven of them summarized in table 1 are produced almost quantitatively, except with dehydrocarolic acid. All of them give either one or two molecules of carbon dioxide. All of them give one molecule of acetoin with the exception of dehydrocarolic acid, from which diacetyl is produced. The distinctive features of the different molecules are seen in the hydrolysis products in the last column—butyrolactone, *l*-*n*-hexanolactone, succinic acid and *n*-butyric acid. This, together with much supporting evidence, justifies we believe the structural formulae assigned to the different tetronic acids. So far none of the seven given in table 1 has been synthesized, except  $\gamma$ -methyltetronic acid.

#### DERIVATIVES OF CITRIC ACID

We now turn to citric acid (XVI) and a number of other fungal metabolic products which are structurally related to it. Citric acid itself has been known to be a mould metabolite for over half a century, and has been reported by numerous workers from a large number of different species in different genera of moulds and from some higher fungi. Several thousand tons of it are now manufactured annually in yields of the order of 90 % of the theoretical by the fermentation of sugar solutions with *A. niger*.

Itaconic acid (XVII) was first isolated by Kinoshita from cultures of *A. itaconicus* in 1931 (33, 34) and in 1939 in my department from *A. terreus* (35). Since then, American workers using different strains of *A. terreus* and modified cultural conditions have reported yields of itaconic acid of the order of 30 % of the theoretical (36).

Spiculisporic acid (XVIII) has been obtained from three different species of *Penicillium*, *P. spiculisporem* (37), *P. crateriforme* (38) and *P. minio-luteum* (39). It may be regarded as a decyl-homocitric acid (40), as is indicated, *inter alia*, by the fact that  $\gamma$ -ketopentadecic acid (XIX) is also a metabolite of *P. spiculisporem* (37) and is formed from spiculisporic acid by oxidation with potassium permanganate.

Minioluteic acid (XX) occurs along with spiculisporic acid in the culture filtrate of *P. minio-luteum* (39). It is a decyl- $\alpha$ -hydroxycitric acid.

Caperatic acid is a monomethyl ester of nor-caperatic acid (XXI), but it is not known at present which of the three carboxyl groups is esterified in caperatic acid itself. It was described as a constituent of the lichen *Parmelia caperata* by Hesse in 1898(41), and the structure of nor-caperatic acid, which is clearly a tetradecyl-citric acid, was established by Asano & Ohta (42, 43).

Agaricic acid (XXII), from the higher fungus *Fomes officinalis* (*Polyporus officinalis*), has been known for a century. It is clearly a hexadecylcitric acid (44).

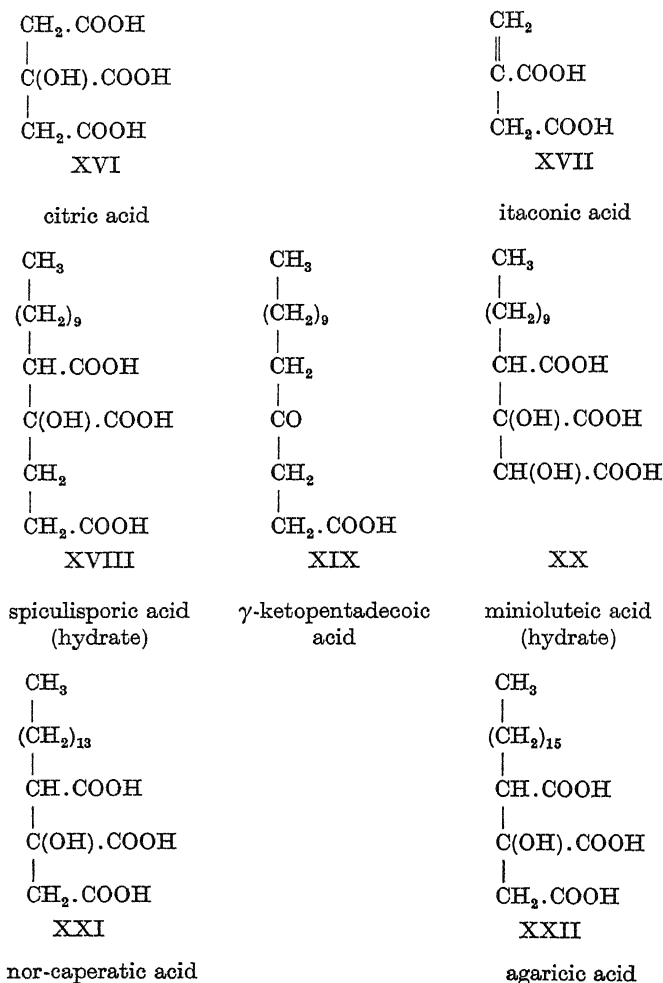
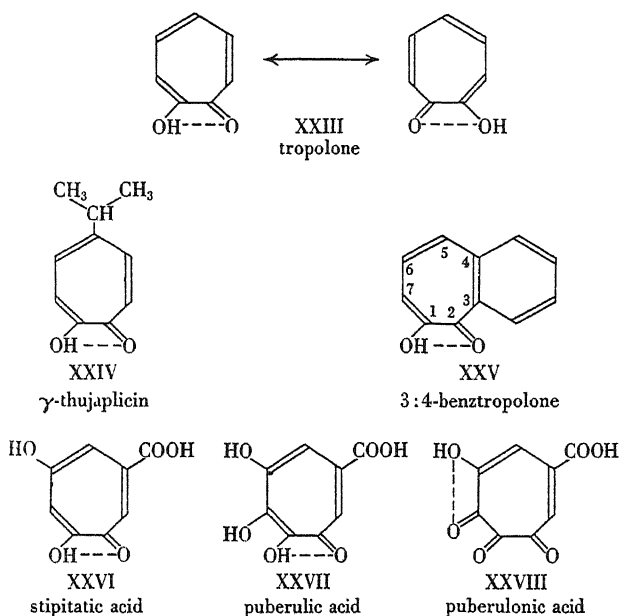


TABLE 2. SUBSTITUTED SUCCINIC ACIDS AS BREAKDOWN PRODUCTS OF MOULD METABOLIC PRODUCTS OR LICHEN ACIDS

mould metabolic product or lichen acid	substituted succinic acid
terrein	<i>d-n</i> -propyl
palitantin	<i>d-n</i> -heptyl
minioluteic acid	<i>n</i> -decyl
nor-caperatic acid	$\alpha$ -methyl- $\alpha'$ - <i>n</i> -tetradecyl
agaricic acid	$\alpha$ -methyl- $\alpha'$ - <i>n</i> -hexadecyl

Before leaving the aliphatic mould metabolites there is a matter of some structural significance to which I wish to draw your attention. In the course of the investigation of their molecular constitution it has been found that a number of products from moulds, higher fungi and lichens give rise to substituted succinic acids on degradation. These are summarized in table 2. Thus tetrahydroterrein gives *n*-propylsuccinic acid (22). Palitantin,  $C_{14}H_{22}O_4$ , a metabolite of *Penicillium palitans*, is a complex unsaturated dihydroxyaldehyde of at present unsettled molecular constitution (45). Its reduction product, tetrahydropalitantin, yields *n*-heptylsuccinic acid on oxidation. The long side-chains in the substituted succinic acids obtained by the breakdown of minioluteic (39), nor-caperatic (43) and agaricic (43) acids contain respectively 10, 14 and 16 carbon atoms.

## DERIVATIVES OF TROPOLONE

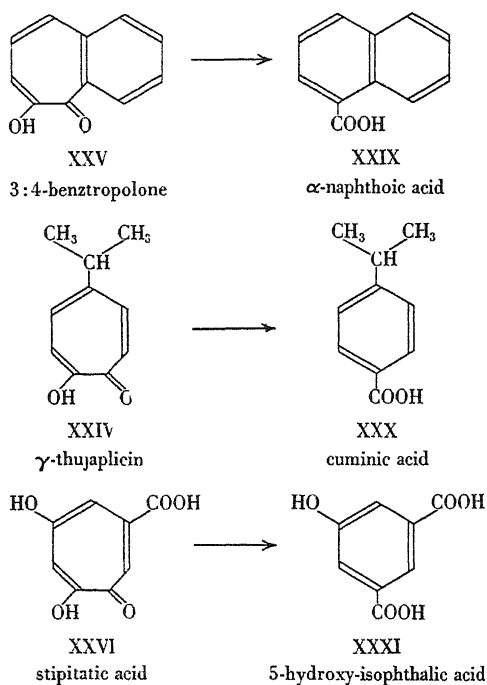


We now come to a group of three quasi-benzenoid mould metabolites, stipitatic acid,  $C_8H_6O_5$ , from *Penicillium stipitatum* (46), and puberulic acid,  $C_8H_6O_6$ , and puberulonic acid,  $C_8H_4O_6$ , from *P. puberulum* (47). A large amount of work was carried out by my colleagues, including the late Professor George Barger (48), and myself without our being able to offer a plausible structural formula for any of them. Thanks, however, to a novel suggestion made in 1945 by Dr M. J. S. Dewar of Oxford it now appears probable that they all contain a 7 carbon ring and are derivatives of what Dr Dewar suggests should be called tropolone (XXIII) (49, 50).

Since 1945 evidence has accumulated supporting Dr Dewar's suggestion. Colchicine is believed to contain a tropolone ring (50, 51, 52). Erdtman & Gripenberg of Stockholm have proved conclusively that  $\alpha$ -,  $\beta$ - and  $\gamma$ -thujaplicins (XXIV) from *Thuja plicata*, the western red cedar tree, are respectively  $\alpha$ -,  $\beta$ - and  $\gamma$ -isopropyl-

tropolones (53, 54). A few weeks ago Professor J. W. Cook of Glasgow announced the synthesis of 3:4-benzotropolone (XXV) (55).

The chemical properties of stipitatic, puberulic and puberulonic acids agree with their formulation as tropolone derivatives. Dr Aulin-Erdtman of Stockholm (private communication) has determined their ultra-violet absorption spectra and concludes from the similarity in spectral type between them and the three thujaplicins that they may all be put into one and the same structural type. The structure assigned to stipitatic acid (XXVI) may, I think, be regarded as a fairly certain one. That suggested for puberulic acid (XXVII) is purely speculative, since there is at present no clear evidence as to the positions occupied by the carboxyl and the two hydroxy groups. Accepting this formulation, however, puberulonic acid may well have the structure XXVIII, since Barger obtained evidence, which unfortunately was not published and is now lost, that puberulonic acid is a quinonoid body closely related to puberulic acid.

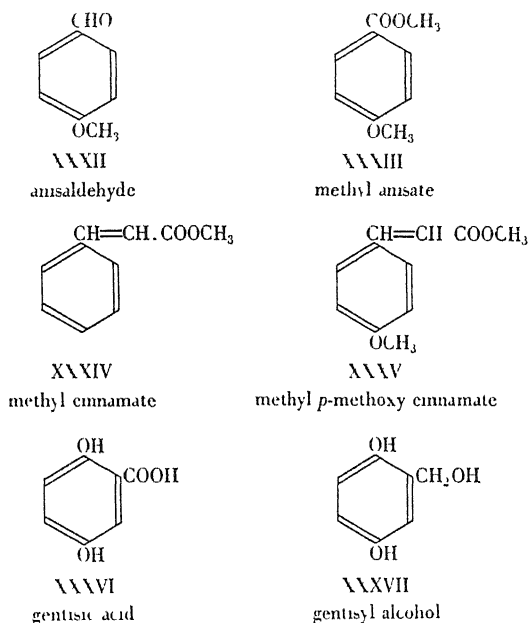


A striking characteristic of the tropolones is the ease with which they are almost quantitatively converted into the corresponding substituted benzoic acids on heating with very strong aqueous potash. Thus 3:4-benzotropolone (XXV) yields  $\alpha$ -naphthoic acid (XXIX) (55),  $\gamma$ -thujaplicin (XXIV) gives cuminic acid (XXX, *p*-isopropyl-benzoic acid) (54), and stipitatic acid (XXVI) gives 5-hydroxy-*iso*-phthalic acid (XXXI) (46). While it is not suggested that this mechanism explains the formation of true benzenoid compounds by moulds, we may regard stipitatic acid as one possible bridge between them and the aliphatic mould metabolites. So let us now examine some of the benzenoid mould metabolites.



## SIMPLE BENZENE COMPOUNDS WITH ONE CARBON CHAIN

My colleague, Dr J. H. Birkinshaw, and Dr W. P. K. Findlay, of the Forest Products Research Laboratory, Princes Risborough, have recently been engaged in a study of the biochemistry of the wood-rotting fungi. They have isolated from some of these higher fungi a number of interesting metabolic products. In particular, they have shown that laboratory cultures of *Trametes suaveolens* and *Lentinus lepideus*, both of which have characteristic aromatic odours, contain the series of four simple benzenoid compounds, XXXII, XXXIII, XXXIV, XXXV, which are clearly closely related to each other structurally. Cultures of *Trametes suaveolens* owe their odour to anisaldehyde (XXXII) and methyl anisate (XXXIII) (56), and those of *Lentinus lepideus* to the methyl esters of cinnamic acid (XXXIV) and *p*-methoxycinnamic acid (XXXV) (57). It will be seen that all four metabolites contain either a methyl ether or a methyl ester grouping or both.

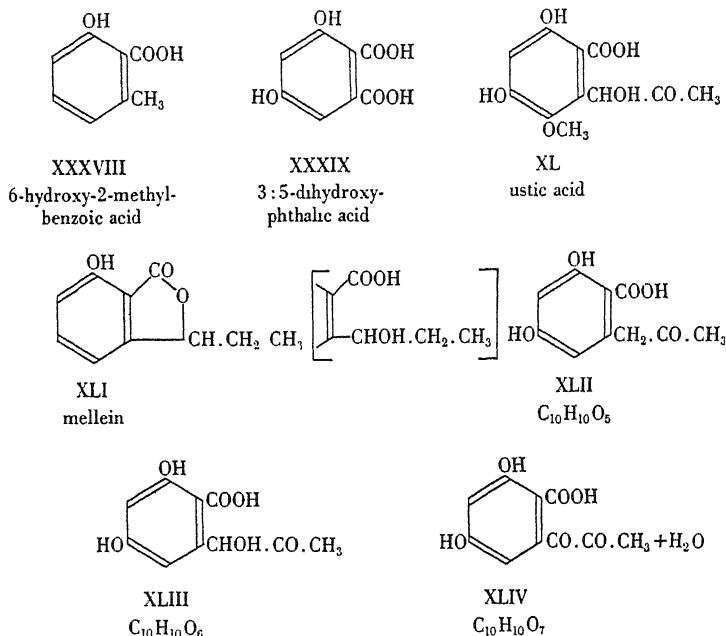


Two other simple benzene compounds with one carbon chain—gentisic acid (XXXVI) and gentisyl alcohol (XXXVII)—are metabolites respectively of *Penicillium griseo-fulvum* (58) and *P. patulum* (59). It is a curious fact that such a simple substance as gentisyl alcohol has not, so far as we can ascertain, been described previously, although the corresponding acid and aldehyde have been known for a long time.

## SIMPLE BENZENE COMPOUNDS WITH TWO CARBON CHAINS

We now come to a group of benzenoid metabolic products, three of which seem to me to be of considerable importance, since, as will become clear later, they appear to represent a stage in the biosynthesis of a number of more complex mould metabolites. The three metabolites to which I wish to draw your attention are 6-hydroxy-2-methylbenzoic acid (XXXVIII) from *P. griseo-fulvum* (60, 58) and *P. flexuosum* (61),

3:5-dihydroxyphthalic acid (XXXIX) from *P. brevi-compactum* (62, 63), and ustic acid (XL) from *Aspergillus ustus* (64). 6-Hydroxy-2-methylbenzoic acid is clearly related to mellein (XLI) from *A. melleus* (65, 66) and from *A. ochraceus* (67, 68), and is, in fact, produced from it by potash fusion. Mellein itself is the lactone of the acid given in

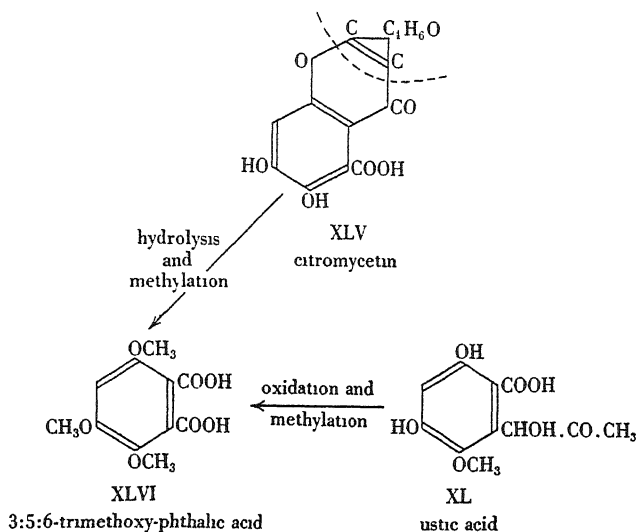


brackets. 3:5-Dihydroxyphthalic acid is also clearly related to the other metabolic products of *Penicillium brevi-compactum*, namely, the acids C<sub>10</sub>H<sub>10</sub>O<sub>5</sub> (XLII), C<sub>10</sub>H<sub>10</sub>O<sub>6</sub> (XLIH) and C<sub>10</sub>H<sub>10</sub>O<sub>7</sub> (XLIV) (62, 69, 70). Each of these three compounds, as well as mellein and ustic acid, has a side-chain of three carbon atoms in varying degrees of oxidation. Ustic acid itself may be regarded as the methoxy derivative of C<sub>10</sub>H<sub>10</sub>O<sub>6</sub>, although it is not yet established with certainty in which of the possible tautomeric forms the three carbon side-chain is present.

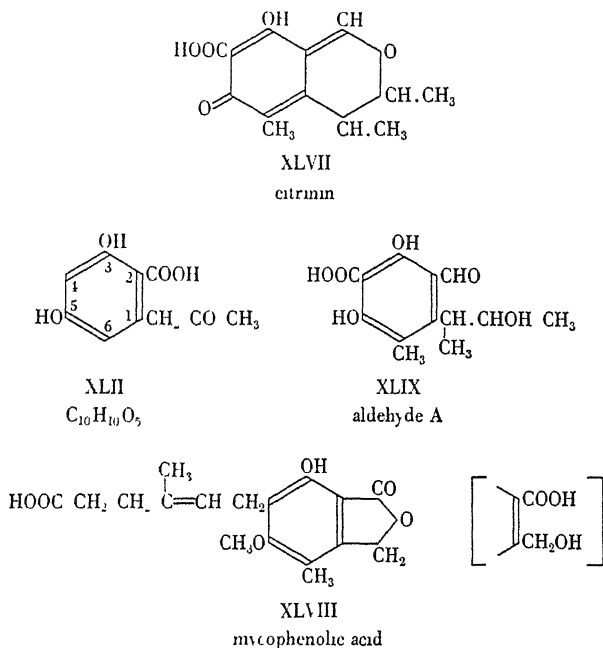
#### CITROMYCETIN

An interesting example of interrelationship in structure is afforded by the mould metabolite citromycetin (XLV).

Citromycetin is a yellow crystalline substance obtained from cultures of a number of species or strains in the *Penicillium frequentans* group (71), some of which were formerly regarded as species of *Citromyces*—hence the name of the metabolite. The complete structure of citromycetin has not yet been established, but a partial structure, in which the nature of the C<sub>4</sub>H<sub>6</sub>O group is at present doubtful, is represented by XLV. Alkaline hydrolysis of the dimethyl ether of citromycetin, followed by methylation of the hydrolysis product, yields 3:5:6-trimethoxyphthalic acid (XLVI) (64). The same compound is formed by the oxidation, followed by methylation of the oxidation product, of the metabolite ustic acid (XL) from *Aspergillus ustus* (64).



## CITRININ, MYCOPHENOLIC ACID



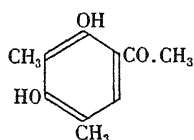
Further examples of interrelationships in structure are afforded by the mould metabolic products citrinin, mycophenolic acid and one of the  $C_{10}$  acids,  $C_{10}H_{10}O_5$ , from *Penicillium brevi-compactum*. Citrinin (XLVII), which is a beautifully crystalline yellow metabolite of *P. citrinum* (72, 73), *Aspergillus terreus* (74) and other moulds, has recently been synthesized by Professor Alexander Robertson and his co-workers at Liverpool (75). The final step in this synthesis was the ring closure of the aldehyde A (XLIX) which, by loss of water and molecular rearrangement, led to the quinonoid body citrinin.

Mycophenolic acid (XLVIII) was first isolated by Alsberg & Black in 1913 from cultures of *Penicillium stoloniferum* (76). Since then it has been reported from a large number of other species and strains in the *P. brevi-compactum* series (62). Mycophenolic acid has not yet been synthesized, but there is strong evidence in support of the structure advanced and shown, in brackets, with the phthalide ring opened (77, 78).

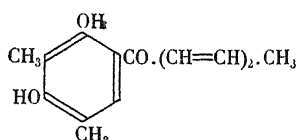
Turning now to the acid  $C_{10}H_{10}O_5$  (XLII) from *P. brevi-compactum* it will be seen that by the introduction of a methyl group into position 6 and a carboxyl group into position 4 we arrive at a structure very similar to that of the aldehyde A from which citrinin was derived. Similarly, by the introduction of a methyl group again into position 6 and the appropriate long side-chain again into position 4 we arrive at a structure very closely related to mycophenolic acid.

There is also a general though not complete similarity in structure between the aldehyde A and two other mould metabolites, clavatol and sorbicillin, which have been described quite recently.

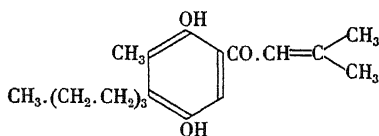
CLAVATOL, SORBICILLIN, FLAVOGLAUCIN, AUROGLAUCIN



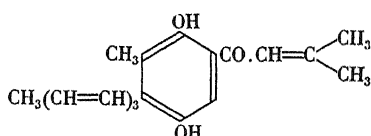
L  
clavatol



LI  
sorbicillin



LII  
flavoglaucin



LIII  
auroglaucin

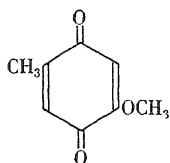
Clavatol (L) was isolated by Bergel *et al.* from culture filtrates of *Aspergillus clavatus* (13). Its structure was determined and its synthesis carried out by Dr C. H. Hassall and Professor A. R. Todd at Cambridge (79).

Sorbicillin (LI) was described by D. J. Cram in America as an orange crystalline metabolic product of *Penicillium notatum*, and was isolated by him from commercial clinical penicillin (80). Hence the possibility is not excluded that sorbicillin is an artefact arising during the manufacturing processes used for the production of penicillin.

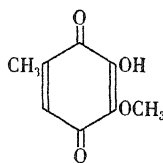
Clavatol and sorbicillin may be regarded as the resorcinol analogues of the substituted quinol metabolites flavoglaucin and auroglaucin, both of which have been isolated from the mycelium of a large number of species in the *Aspergillus glaucus* series (81). The general nature of the structures of the yellow flavoglaucin and the orange-red auroglaucin as substituted quinols was established in the Dyson Perrins Laboratory, Oxford (82, 83), although the exact position and nature of the

substituent side-chains was not determined with certainty. Quite recently Italian workers (84) have confirmed the general correctness of this work and have produced evidence that flavoglaucin has structure LII and auroglaucin structure LIII. Flavoglaucin and auroglaucin are thus clearly derivatives of toluquinol, so that we proceed logically to the consideration of mould metabolic products which are derivatives of toluquinone.

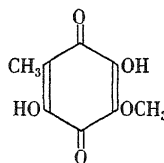
## DERIVATIVES OF TOLUQUINONE



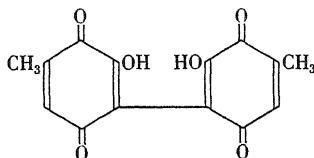
LIV  
4-methoxy-2:5-toluquinone



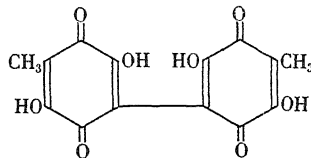
LV  
fumigatin



LVI  
spinulosin



LVII  
phoenicin



LVIII  
oosporein

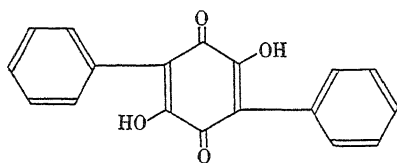
In recent years moulds have proved to be very fruitful sources of a number of different types of quinones. Many moulds are highly coloured—yellow, orange, red, purple and violet—and these colours have frequently been shown to be due to the presence of quinones or their salts, depending on the pH of the medium.

The first group of quinones which I wish to discuss is the series of five derivatives of toluquinone given above. All of them have been synthesized and they form a perfect series of structurally interrelated metabolites.

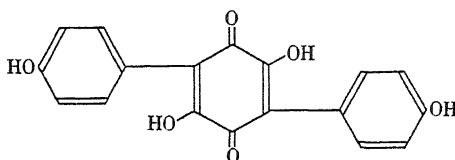
4-Methoxy-2:5-toluquinone (LIV) which is orange yellow in colour was reported a few months ago by workers in the New York Botanical Gardens as a metabolite of laboratory cultures of the higher fungi *Coprinus similis* and *Lentinus degener* (85). Fumigatin (LV), maroon red in colour, occurs in the culture filtrates of an unusual but authentic strain of *Aspergillus fumigatus* (86). It is clearly 3-hydroxy-4-methoxy-2:5-toluquinone (87). Spinulosin (LVI), deep purple black in colour, was first isolated from cultures of *Penicillium spinulosum* (88), and later from a different strain of *Aspergillus fumigatus* (89) from that which gave fumigatin, and quite recently from *Penicillium cinerascens* (26). Spinulosin is clearly 6-hydroxyfumigatin (86, 90).

Phoenicin (LVII) and oosporein (LVIII) are derivatives of 4:4'-ditoluquinone. It will be seen that phoenicin bears the same relationship to fumigatin as does oosporein to spinulosin. Phoenicin was discovered in 1933 by Dr E. Friedheim in Switzerland as a metabolic product of *P. phoeniceum* (91, 92), and its constitution and synthesis were described by Dr T. Posternak (93) who also isolated it from

*P. rubrum* (94). Oosporein was obtained by Kögl & van Wessem in Holland in 1944 from laboratory cultures of the mould *Oospora colorans* (95).



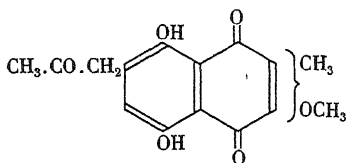
LIX  
polyporic acid



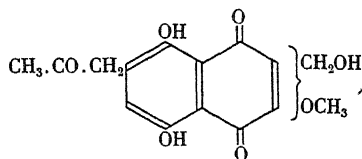
LX  
atromentin

Spinulosin (LVI) is also closely related to a number of other naturally occurring derivatives of benzoquinone, and in particular to two substances investigated by Kögl and his co-workers and isolated by them from naturally occurring specimens of two of the higher fungi. These fungal metabolic products are polyporic acid (LIX) from *Polyporus nidulans* (96), and atromentin (LX) from *Paxillus atrotomentosus* (97). It will be seen that the methyl and methoxy groups in spinulosin are replaced by two phenyl groups in polyporic acid, and by two *para*-hydroxyphenyl groups in atromentin.

#### DERIVATIVES OF NAPHTHAQUINONE



LXI  
javanicin

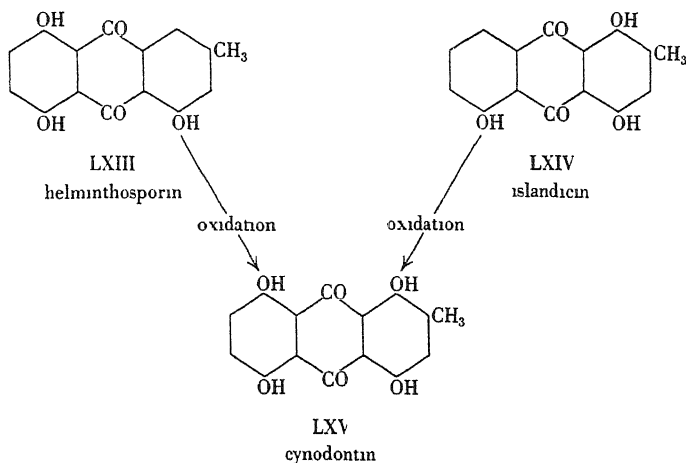


LXII  
oxyjavanicin

So far as I have been able to ascertain only two substituted naphthaquinones have been isolated from cultures of moulds, namely, javanicin,  $C_{15}H_{14}O_6$  (LXI), and oxyjavanicin,  $C_{15}H_{14}O_7$  (LXII). These two substances were prepared from the

blood-red culture filtrate of *Fusarium javanicum* by Dr A. H. Cook and his colleagues at Imperial College, London (98, 99). The molecular structure of these compounds has not yet been completely established with certainty, but, as a result of analytical work and the determination of absorption spectra, javanicin is believed to have the partial structure given above. Oxyjavanicin would then have a similar structure except that a hydroxymethyl group would take the place of the methyl group in javanicin. In the absence of complete molecular structures one can only remark that javanicin contains a methyl and methoxy group as do fumigatin (LV) and spinulosin (LVI), and a  $\text{CH}_3\text{CO} \cdot \text{CH}_2$  side-chain as does the  $\text{C}_{10}\text{H}_{10}\text{O}_5$  acid (XLII) from *Penicillium brevi-compactum*.

## DERIVATIVES OF ANTHRAQUINONE



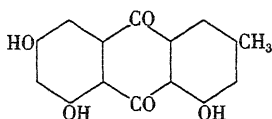
Since 1933 a considerable number of polyhydroxyanthraquinones, previously unknown to organic chemistry, have been shown to be metabolites of different species in a number of different genera of moulds. They are often produced in considerable amounts. Thus the dried mycelium of *Helminthosporium gramineum* contained 30 % of its weight of a mixture of polyhydroxyanthraquinones (100).

The first one which we isolated was helminthosporin (LXIII) from the mycelium of *H. gramineum* (100), *H. catenarium* and *H. tritici-vulgaris* (101) and *H. cynodontis* (102), and I welcome the opportunity of acknowledging publicly my indebtedness to our President for his guidance and collaboration in elucidating the structure of many of the anthraquinones which I shall describe, since he was associated with this work from the earliest days of the isolation of helminthosporin.

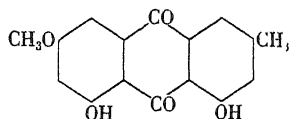
Islandicin (LXIV) was isolated from cultures of *Penicillium islandicum* (103), and cynodontin (LXV) from *Helminthosporium cynodontis* (102), *H. euchlaenae* (102) and *H. avenae* (101). Helminthosporin (104, 105) and cynodontin (106) have been synthesized.

Helminthosporin and islandicin are both readily converted into cynodontin by oxidation with manganese dioxide and concentrated sulphuric acid, so that the close structural relationship between these three substances is evident. Islandicin is also closely related to catenarin (LXXII) and erythroglaucin (LXXIII), which are respectively 7-hydroxy- and 7-methoxy-islandicin.

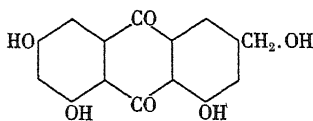
An outstanding feature of many of the anthraquinone mould metabolites is their close structural relationship to *Frangula* emodin. So far as I am aware emodin itself (LXVI) has not been found in any mould, though Kögl isolated it in 1925 from natural specimens of the higher fungus *Dermocybe sanguinea* (107). Physcion (LXVII), the 7-methyl ether of emodin, has long been known as a lichen constituent (108, 109) and has also been isolated from laboratory cultures of seventeen species or strains in the *Aspergillus glaucus* series (82, 110, 111). The previously undescribed  $\omega$ -hydroxyemodin (LXVIII) was isolated independently and almost simultaneously by ourselves from *Penicillium cyclopium* (112), and by Posternak from *P. citreo-roseum* and was called by him citreorosein (LXVIII) (113, 114). The 4-methyl ether of



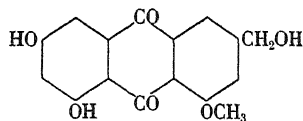
LXVI  
emodin



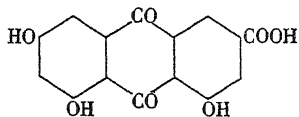
LXVII  
physcion



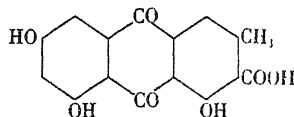
LXVIII  
 $\omega$ -hydroxyemodin  
citreorosein



LXIX  
carviolin  
roseopurpurin



LXX  
emodic acid



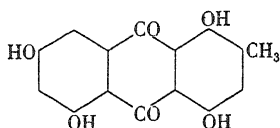
LXXI  
endocrocin

$\omega$ -hydroxyemodin was also described independently and almost simultaneously by Hind from *P. carmino-violaceum* (115, 116) and called by him carviolin (LXIX), and by Posternak from *P. roseopurpureum* (117, 118) and called by him roseopurpurin (LXIX). Emodic acid (LXX) occurs along with  $\omega$ -hydroxyemodin in cultures of *P. cyclopium* (112). Endocrocin (LXXI), which is 3-carboxyemodin, was obtained by Asahina & Fuzikawa from the Japanese lichen, *Nephromopsis endocrocea* (119). It will be noticed that all these six compounds may be regarded as being derived from 3:5-dihydroxyphthalic acid (XXXIX), which, as I have shown previously, is a metabolite of *Penicillium brevi-compactum* (63), and that endocrocin might, on paper at any rate, be quite easily synthesized by coupling 3:5-dihydroxyphthalic acid with the metabolite of *P. griseo-fulvum*, 6-hydroxy-2-methylbenzoic acid (XXXVIII) (60, 61).

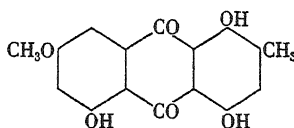
Three other anthraquinones, each of which may be regarded as a derivative of emodin, and each of which contains four nuclear hydroxyl groups, are shown on p. 497.



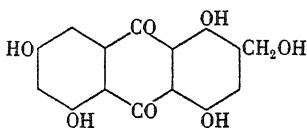
Catenarin (LXXII) from *Helminthosporium catenarium* (101), *H. gramineum* (100), *H. velutinum* and *H. tritici-vulgaris* (101) is clearly 1-hydroxyemodin (120). Erythroglaucin (LXXIII), from fifteen species or strains in the *Aspergillus glaucus* series (111), is clearly the 7-methyl ether of catenarin (120). The molecular constitutions of catenarin and erythroglaucin have been confirmed by synthesis (121, 120), but that of tritisporin (LXXIV) from *Helminthosporium tritici-vulgaris*, although quite probable, is less firmly established (101). Accepting it, however, tritisporin becomes 1-hydroxy- $\omega$ -hydroxyemodin.



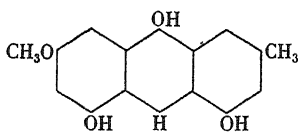
LXXII  
catenarin



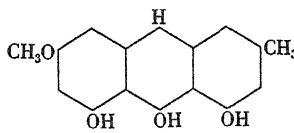
LXXIII  
erythroglaucin



LXXIV  
tritisporin



LXXV  
physcion anthranol A



LXXVI  
physcion anthranol B

Catenarin, erythroglaucin and tritisporin can obviously be built up from the mould metabolite 3:5-dihydroxyphthalic acid (XXXIX) (63), coupled, in the case of tritisporin, with a second mould metabolite, gentisyl alcohol (XXXVII) (59), and in the other two cases with toluquinol, which while not yet described as a mould metabolite may be confidently expected to be one in view of its close relationship to gentisyl alcohol.

The close relationship to physcion and hence to emodin of the two anthranols of physcion, A (LXXV) and B (LXXVI), which come from a few species in the *Aspergillus glaucus* series (111), is obvious.\*

A number of mould-colouring matters, steadily increasing in number and having high melting-points, are almost certainly polyhydroxy di-anthraquinones, and some of them are clearly derivatives of emodin. For that reason I think that they are

\* It will be seen that, without exception, all the anthraquinone metabolites which I have described are derivatives of chrysophanol (chrysophanic acid), 4:5-dihydroxy-2-methylantraquinone. The observation, made by Mr B. H. Howard in my department since this lecture was delivered, that chrysophanol is one of the colouring matters of *Penicillium islandicum* is therefore of considerable interest.

worthy of consideration here, in spite of the fact that their molecular constitution has not yet been completely established.

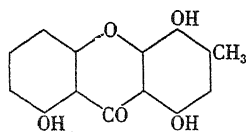
Penicilliopsisin from the mould *Penicilliopsis clavariaeformis*<sup>(122)</sup> has the empirical formula  $C_{30}H_{24}O_8$  and melts at about  $330^\circ C$ . On thermal decomposition it yields emodin anthranol, and on oxidation with nitric acid it gives tetranitro-emodin.

A pigment from *Penicillium islandicum*, *P. wortmanni* and *P. rugulosum* which we are investigating at present has the probable empirical formula  $C_{31}H_{20}O_{11}$ , and does not melt below  $390^\circ C$ . On treatment with cold aqueous sodium hyposulphite,  $Na_2S_2O_4$ , in sodium carbonate solution it gives 90 % of the theoretical yield of two molecules of emodin, a curious if not indeed an almost unique reaction.

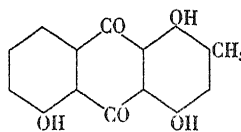
Rugulosin,  $C_{22}H_{20}O_8$  (?), m.p.  $294^\circ C$ , from a large number of different strains of *P. rugulosum*, is not itself an anthraquinone, but is, we believe, a complex and highly substituted benzoyl benzoic acid (unpublished observations). However, it appears to be related to the anthraquinones since, on thermal decomposition, it gives about 30 % of a mixture of emodin and chrysophanol.

Finally, aurofusarin from *Fusarium culmorum*<sup>(123)</sup>, has the empirical formula  $C_{30}H_{20}O_{12}$  and does not melt below  $360^\circ C$ . Little is known of its constitution except that it contains two methoxy groups and that its general properties are those of a polyhydroxy di-anthraquinone.

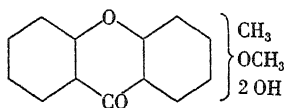
#### DERIVATIVES OF XANTHONE



LXXVII  
ravenelin



LXIV  
islandicin



LXXVIII  
rubrofusarin

Two xanthone derivatives are known as mould metabolites.

Ravenelin (LXXVII) was isolated from the mycelium of laboratory cultures of *Helminthosporium ravenelii* and *H. turcicum*<sup>(124)</sup>. Its molecular structure is shown above, and this was subsequently confirmed by its synthesis in 1944 by Mull & Nord<sup>(125)</sup>. Its very close structural relationship to the anthraquinone metabolite islandicin (LXIV) from *Penicillium islandicum* is obvious.

Rubrofusarin (LXXVIII) occurs along with the di-anthraquinone derivative aurofusarin in the mycelium of *Fusarium culmorum*<sup>(123)</sup>. It is the monomethyl ether of a methyl trihydroxyxanthone which is isomeric but not identical with ravenelin. Its molecular constitution has not yet been established with certainty, although Mull & Nord suggest two highly speculative structures<sup>(125)</sup>.

The last group of mould metabolites which I wish to discuss is chosen not so much because the members of it are structurally related as because they all contain chlorine.

TABLE 3. MOULD METABOLITES CONTAINING CHLORINE

metabolite	empirical formula	species of micro-organism	investigators	reference
caldariomycin	$C_5H_8O_2Cl_2$	<i>Caldariomyces fumago</i>	London School of Hygiene and Tropical Medicine	(126)
chloromycetin	$C_{11}H_{12}O_5N_2Cl_2$	<i>Streptomyces venezuelae</i>	Parke, Davis Labs.	(127), (128)
erdin	$C_{16}H_{10}O_7Cl_2$	<i>Aspergillus terreus</i>	L.S.H.T.M.	(129)
geodin	$C_{17}H_{12}O_7Cl_2$	<i>Aspergillus terreus</i>	L.S.H.T.M.	(129)
griseofulvin	$C_{17}H_{17}O_6Cl$	<i>Penicillium griseo-fulvum</i> <i>P. janczewskii</i>	L.S.H.T.M.	(130)
sclerotiorine	$C_{20}H_{19(21)}O_5Cl$	<i>P. sclerotiorum</i>	Brian, Curtis & Hemming	(131), (132), (133)
aureomycin	$C_{22}H_{27}O_8N_2Cl(?)^*$	<i>Streptomyces aureofaciens</i>	Curtin & Reilly Lederle Labs.	(134) (135), (136)

\* This empirical formula has not been published but is one of the possible formulae calculated from data given in reference (136).

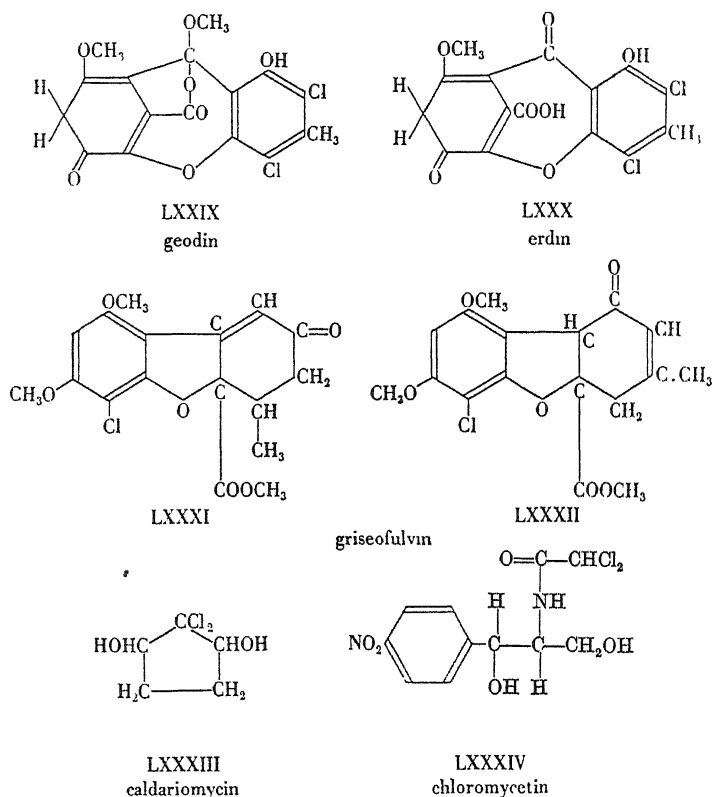
It will be remembered that one of our standard media—Czapek-Dox medium (p. 482)—contains, in addition to glucose, only mineral salts which include half a gram of potassium chloride per litre of medium. A quantitative survey, carried out in the late 1930's, of the chlorine metabolism of 139 species or strains of moulds grown on this medium, revealed the fact that certain species metabolize the inorganic chlorine ion and convert it into non-ionic chlorine (126). This observation, among others which we had made previously, led us to the isolation of four new metabolic products containing organically bound chlorine, namely, caldariomycin from *Caldariomyces fumago*, erdin and geodin from *Aspergillus terreus*, and griseofulvin from *Penicillium griseo-fulvum*. These were followed by the isolation in other laboratories of sclerotiorine from *P. sclerotiorum*, and, quite recently, by chloromycetin and aureomycin from two different species of *Streptomyces*.

All of these seven metabolites are crystalline substances, and chloromycetin and aureomycin, both of which contain nitrogen in addition to chlorine, are the new antibiotics which are unique in having very powerful anti-rickettsial and anti-viral properties. Chloromycetin is the only one which has been synthesized (137), but structural formulae have been advanced for all of them except sclerotiorine and aureomycin. These structural formulae are given below.

Geodin (LXXIX) and erdin (LXXX) are closely related structurally to each other (138, 139), and, thanks to a suggestion of Professor A.R. Todd and Dr Hassall, we now regard geodin, which is optically active, as the pseudo-methyl ester of erdin, which is optically inactive (140).

Two structural formulae (LXXXI and LXXXII) have been proposed for griseofulvin. The original structure (LXXXI) was advanced by ourselves (130), while structure LXXXII was proposed later by Grove & McGowan (133).

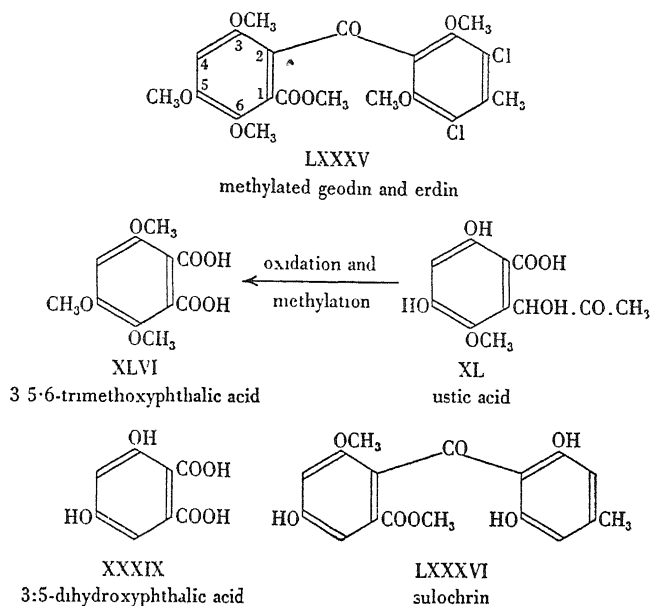
Caldariomycin (LXXXIII) was, until recently, the only chlorine containing metabolite having two chlorine atoms attached to the same carbon atom (126). A similar grouping is now known to be present in chloromycetin (LXXXIV) which, on acid or alkaline hydrolysis, yields, *inter alia*, dichloroacetic acid. Chloromycetin is unique among microbial metabolites and also I believe among any natural products in containing a nitrobenzene grouping. The antibiotic is the D-(-)-threo form (137).



Geodin (LXXIX) and erdin (LXXX) are closely related structurally to at least three other mould metabolites. On methylation with methyl sulphate and alkali, the seven-membered ring opens and there is formed from both geodin and erdin the same highly substituted benzoyl benzoic acid shown in structure LXXXV (140). This compound can clearly be derived from 3:5:6-trimethoxyphthalic acid (XLVI) which is itself formed by the oxidation and methylation of the mould metabolite ustic acid (XL) from *Aspergillus ustus* (64), and by hydrolysis and methylation of citromycetin (XLV) from *Penicillium frequentans* (71).

Further, by removal of the two chlorine atoms in the right-hand ring and of the methoxy group in position 6 in the left-hand ring, we arrive at the same basic

structure as is present in sulochrin (LXXXVI), a metabolic product of the mould *Oospora sulphurea-ochracea* described by Nishikawa (141, 142, 143), and sulochrin in its turn may be derived from 3:5-dihydroxyphthalic acid (XXXIX), a metabolite of *Penicillium brevi-compactum* (63).



#### BIOLOGICAL SIGNIFICANCE OF MOULD METABOLIC PRODUCTS

The large number of mould metabolic products and, from the chemical point of view, their great variety in structure naturally prompts the question as to what function they serve in the economy of the organisms which produce them.

In trying to answer this question, though in a negative sense, I wish to emphasize that it is my opinion that they cannot, in general, be regarded as by-products of no particular biological importance. If this were so why should many of them be produced in such considerable yields? Further, it should be remembered that these large yields can often only be obtained by harvesting the cultures before all the glucose in the medium has been completely metabolized. Otherwise long-continued incubation leads to their complete disappearance by oxidation to carbon dioxide and water.

There is a growing belief that certain types of metabolites play some part in the oxidation-reduction mechanisms of the moulds which produce them. Thus the culture filtrates of *Aspergillus fumigatus* from which fumigatin (LV) was isolated also contain the corresponding quinol, 3-hydroxy-4-methoxytoluquinol (86). Similarly phenicin (LVII) is accompanied by its leuco derivative, tetrahydrophenicin, in cultures of *Penicillium rubrum* (94); physcion (LXVII) occurs in the mycelium of certain species in the *Aspergillus glaucus* series along with its two reduction products, i.e. the anthranols 4:5-dihydroxy-7-methoxy-2-methyl-9-anthranol (LXXV) and 4:5-dihydroxy-7-methoxy-2-methyl-10-anthranol (LXXVI) (111); the mycelium of

*Helminthosporium leersii* contains the yellow luteoleersin,  $C_{26}H_{38}O_7$ , and its colourless reduction product alboleersin,  $C_{26}H_{40}O_7$ , of at present undetermined molecular constitution (144); and culture filtrates of the mould *Oidiodendron fuscum* contain, in addition to the orange crystalline fuscin,  $C_{15}H_{16}O_5$ , its leuco derivative dihydro-fuscin,  $C_{15}H_{18}O_5$  (145). All these quinonoid metabolic products and their corresponding leuco derivatives have been shown to be readily inter-convertible *in vitro*, and Friedheim has shown that traces of phoenicin increase by 200 to 300 % the respiration of unpigmented and washed cells of *Bacillus pyocyaneus* (91, 92).

It seems probable that some of the mould metabolites already described and certainly others which await isolation play a fundamental role in the biological phenomenon known as antibiosis. Thus in addition to those antibiotics of established clinical importance such as penicillin, streptomycin, aureomycin and chloromycetin, a large number of other mould metabolites exhibit very powerful antibacterial and, *significantly*, antifungal properties *in vitro*. This subject has been adequately reviewed recently by Oxford (146), Birkinshaw (147) and Kavanagh (148). The general biological significance, as distinct from the medical importance, of this aspect of the subject is indicated by the following examples.

(a) Van Luijk (149) showed in 1938 that sterilized culture filtrates of the saprophytic mould *Penicillium expansum* totally inhibited the growth *in vitro* of the plant pathogenic fungus *Pythium de Baryanum* at dilutions of 1:1,280, and he obtained good results in suppressing the attack of the *Pythium* on lucerne seedlings by treating infected soil with the active filtrate. The active principle responsible for this effect, patulin (V), was isolated by ourselves from *Penicillium expansum* in 1943 (150, 151) and independently by Dutch workers from a different strain of the same species (16, 17). They named it expansin. It inhibits completely the growth of a number of species of *Pythium* at a concentration of about 1:500,000 (150).

(b) Rayner (152) and Neilson Jones (153) have shown that the growth failure of conifers at Wareham Heath, Dorset, is associated with the development in the soil of a definite toxicity, of biological origin, to the mycorrhizal fungi normally associated with these trees. Brian and his colleagues (154) have demonstrated that moulds which are commonly abundant in meadow or arable soils, e.g. *Mucor* spp., *Trichoderma viride*, *Penicillium chrysogenum* and *Fusarium* spp., are virtually absent in Wareham Heath soil, in which the mould flora is almost restricted to three groups of *Penicillium* spp., viz. strains of *P. janczewskii*, *P. terlikowskii* and strains in the *P. nigricans-janczewskii* series. They have also shown that each of these three groups gives rise to metabolic products which have been isolated in a state of purity and which, at a very low concentration, have marked biological effects on other fungi. Thus, *P. janczewskii* was shown to produce the chlorine containing metabolite griseofulvin (LXXXI and LXXXII) to which they gave the name 'curling factor' since, at concentrations as low as  $1\mu\text{g./ml.}$  it causes excessive branching and distortion of the germ-tubes and hyphae of *Botrytis allii* (131, 132, 133); *P. terlikowskii* produces gliotoxin,  $C_{13}H_{14}O_4N_2S_2$ , first isolated as a colourless crystalline metabolite of *Trichoderma viride* by Weindling & Emerson (155) which, in addition to being actively antibacterial, is also highly fungistatic to a number of saprophytic and plant pathogenic fungi (156); and strains in the *Penicillium nigricans-*

*janczewskii* series yield a new unnamed metabolic product crystallizing in glistening red needles and having the probable molecular formula  $C_{10}H_8O_4$ . This substance is actively fungistatic and prevents the germination of conidia of *Botrytis allii* at concentrations of 0.4  $\mu$ g./ml. at pH 3.5 (157).

### CONCLUSION

My purpose in this lecture has been to present to you, although unavoidably in a very condensed and incomplete form, the relationships in chemical structure between members of representative classes of fungal metabolic products. For many years after the commencement of our work no such relationship was evident, and the only obvious result of it was the accumulation of a number of new natural products, which while scientifically interesting in themselves, as I think all natural products must be to the chemist and biochemist, seemed to have no obvious place in the biochemistry of living organisms.

I think that this was inevitable in the early days of the new subject of mycological chemistry, since it dealt with a group of organisms which had been sadly neglected by botanists except for a small number of mycologists, and which were in general regarded as a nuisance by bacteriologists, and were almost completely ignored by chemists and biochemists. However, I think it can now rightly be claimed that there are signs of order and of a general underlying design in the biochemistry of the fungi, and my personal view of the mould metabolic products is that they are slowly taking their places in the intricate jigsaw puzzle of natural products.

Thus the slight variations in chemical structure which I have described between some of the benzenoid and quinonoid metabolic products of species of fungi which are closely related morphologically to each other are very reminiscent of similar structural relationships in the colouring matters of flowers which have been shown so clearly by the work of Sir Robert and Lady Robinson and Miss Scott-Moncrieff. This is so much the case that I am convinced that by the collaboration of the geneticist and the mycological chemist which is already beginning to take shape, similar laws will be found to hold with the fungi as have been demonstrated clearly in other living organisms.

Thus J. B. S. Haldane has frequently expressed the view that while biochemical differences between different species of organisms are often striking, the differences between members of a single species—call them strains or mutants, or what you will—may in some ways be an even more fruitful field. I share Haldane's view and firmly believe that by the careful investigation of the metabolic products of closely related species, or of strains or mutants of a single species of fungus, grown under controlled and comparable conditions, much may be learned of the mechanism of intermediate metabolism of these organisms. This line of approach is free from the serious objections to the methods which have been much more favoured such as the so-called fixation method, for example by the addition of sulphite or dimedon to fix acetaldehyde in alcoholic fermentation, and the method of selectively poisoning parts of the catalytic system by the use of specific enzyme poisons like sodium fluoride and iodoacetic acid.

## APPENDIX

*List of species cited in text with authorities*Species of *Aspergillus*

- |                                |   |
|--------------------------------|---|
| <i>A. clavatus</i> Desmazières | <i>A. ochraceus</i> Wilhelm             |
| <i>A. fumigatus</i> Fresenius  | <i>A. oryzae</i> (Ahlburg) Cohn         |
| <i>A. glaucus</i> Link         | <i>A. terreus</i> Thom                  |
| <i>A. itaconicus</i> Kinoshita | <i>A. ustus</i> (Bainier) Thom & Church |
| <i>A. melleus</i> Yukawa       | <i>A. wentii</i> Wehmer                 |
| <i>A. niger</i> van Tieghem    |   |

Species of *Penicillium*

- |                                     |                                   |
|-------------------------------------|-----------------------------------|
| <i>P. brevi-compactum</i> Dierckx   | <i>P. nigricans</i> Bainier       |
| <i>P. carmino-violaceum</i> Dierckx | <i>P. notatum</i> Westling        |
| <i>P. charlesii</i> G. Smith        | <i>P. palitans</i> Westling       |
| <i>P. chrysogenum</i> Thom          | <i>P. patulum</i> Bainier         |
| <i>P. cinerascens</i> Biourge       | <i>P. phoeniceum</i> van Beyma    |
| <i>P. citreo-roseum</i> Dierckx     | <i>P. puberulum</i> Bainier       |
| <i>P. citrinum</i> Thom             | <i>P. roseo-purpureum</i> Dierckx |
| <i>P. claviforme</i> Bainier        | <i>P. rubrum</i> Grasberger-Stoll |
| <i>P. crateriforme</i> Abbott       | <i>P. rugulosum</i> Thom          |
| <i>P. cyclopinum</i> Westling       | <i>P. sclerotiorum</i> van Beyma  |
| <i>P. expansum</i> (Link) Thom      | <i>P. spiculisporem</i> Lehman    |
| <i>P. flexuosum</i> Dale            | <i>P. spinulosum</i> Thom         |
| <i>P. frequentans</i> Westling      | <i>P. stipitatum</i> Thom         |
| <i>P. griseo-fulvum</i> Dierckx     | <i>P. stoloniferum</i> Thom       |
| <i>P. islandicum</i> Sopp           | <i>P. terrestre</i> Jensen        |
| <i>P. janczewskii</i> Zaleski       | <i>P. terlukowskii</i> Zaleski    |
| <i>P. minio-luteum</i> Dierckx      | <i>P. wortmanni</i> Klocker       |

Species of *Helminthosporium*

- |                                 |                                     |
|---------------------------------|-------------------------------------|
| <i>H. avenae</i> Eidam          | <i>H. leersii</i> Atkinson          |
| <i>H. catenarium</i> Drechsler  | <i>H. ravenelii</i> Curtis          |
| <i>H. cynodontis</i> Marignoni  | <i>H. tritici-vulgaris</i> Nisikado |
| <i>H. euchlaenae</i> Zimmermann | <i>H. turcicum</i> Passerini        |
| <i>H. gramineum</i> Rabenhorst  | <i>H. velutinum</i> Link            |

## Miscellaneous species

- |  |   |
|--|---|
| <i>Botrytis allii</i> Munn                   | <i>Penicillium clavariaeformis</i> Solms-Laubach  |
| <i>Caldariomyces fumago</i> Woronichin       | <i>Pythium de Baryanum</i> Hesse                  |
| <i>Fusarium culmorum</i> (W. G. Smith) Sacc. | <i>Streptomyces aureofaciens</i> Duggar           |
| <i>Fusarium javanicum</i> Koorders           | <i>Streptomyces venezuelae</i> Ehrlich, Gottlieb, |
| <i>Oidiodendron fuscum</i> Robak             | Burkholder, Anderson & Pridham                    |
| <i>Oospora colorans</i> van Beyma            | <i>Trichoderma viride</i> Persoon ex Fries        |
| <i>Oospora sulphurea-ochracea</i> van Beyma  |   |

## Higher fungi

- |   |   |
|---|---|
| <i>Coprinus similis</i> Berkeley & Broome               | <i>Lentinus lepideus</i> Fries                |
| <i>Cortinarius (Dermocybe) sanguineus</i> (Wulf.) Fries | <i>Paxillus atrotomentosus</i> (Batsch) Fries |
| <i>Fomes officinalis</i> (Villars) Neumann              | <i>Polyporus nidulans</i> Pers. ex Fries      |
| <i>Lentinus degener</i> Kalchbrenner                    | <i>Trametes suaveolens</i> (Linn.) Fries      |

## Lichens

- |  |  |
|--|--|
| <i>Parmelia caperata</i> (Linn.) Acheson | <i>Nephromopsis endocrocea</i> Asahina |
|--|--|





FIGURE 1



FIGURE 2



FIGURE 3



FIGURE 4



FIGURE 5

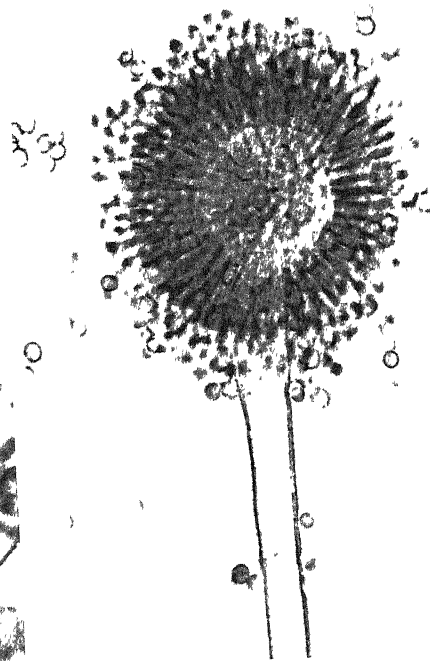


FIGURE 7



FIGURE 6

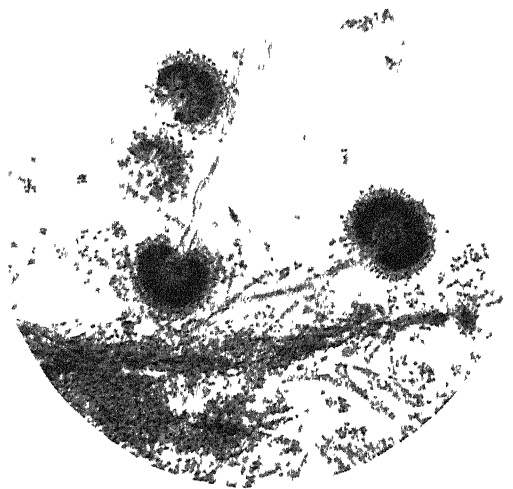


FIGURE 8



FIGURE 9



FIGURE 10



FIGURE 11

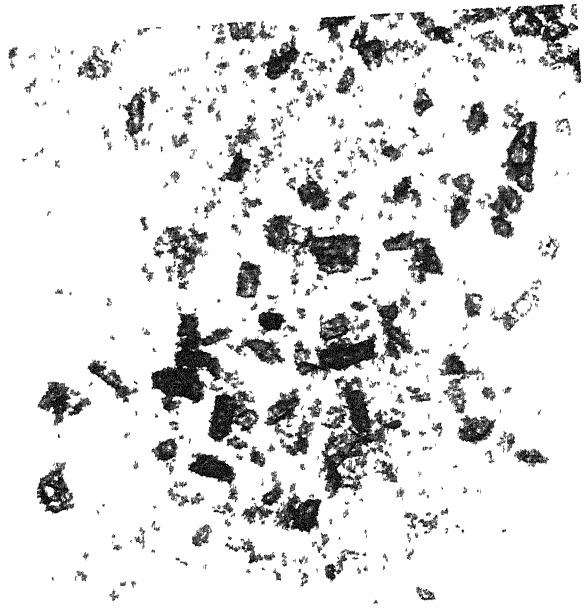


FIGURE 12



FIGURE 13

## DESCRIPTION OF PLATES 23 TO 26

I am much indebted to my colleague, Mr George Smith, London School of Hygiene and Tropical Medicine, for the following photomicrographs of some moulds referred to in the text.

## PLATE 23

FIGURE 1. *Penicillium brevi-compactum* Dierckx (magn.  $\times 600$ ). Many species in this series produce 3:5-dihydroxyphthalic acid, mycophenolic acid, and the acids  $C_{10}H_{10}O_5$ ,  $C_{10}H_{10}O_6$  and  $C_{10}H_{10}O_7$ .

FIGURE 2. *Penicillium charlesii* G. Smith (magn.  $\times 500$ ). Source of the tetronic acids,  $\gamma$ -methyl-tetronic, carolic, carlic, carolinic and carlosic acids.

FIGURE 3. *Penicillium spinulosum* Thom (magn.  $\times 500$ ). Source of spinulosin.

FIGURE 4. *Penicillium citrinum* Thom (magn.  $\times 500$ ). Source of citrinin.

## PLATE 24

FIGURE 5. *Aspergillus chevalieri* (Mangin) var. *intermedius* Thom & Raper (*A. glaucus* series) (magn.  $\times 500$ ). Many species in this series produce the colouring matters flavoglaucin, auroglaucin, erythroglaucin, physcion and the physcion anthranols A and B.

FIGURE 6. *Aspergillus fumigatus* Fresenius (magn.  $\times 250$ ). Source of fumigatin.

FIGURE 7. *Aspergillus flavus* Link (magn.  $\times 500$ ). Source of kojic acid.

FIGURE 8. *Aspergillus terreus* Thom (magn.  $\times 250$ ). Different strains of this species produce terrein, itaconic acid, citrinin, geodin and erdin.

## PLATE 25

FIGURE 9. *Fusarium* sp. Typical conidia (magn.  $\times 900$ ).

FIGURE 10. *Helminthosporium monoceras* Drechsler (magn.  $\times 90$ ).

FIGURE 11. *Helminthosporium gramineum* Rabenhorst (magn.  $\times 250$ ). Showing crystals of colouring matters in mycelium. Source of helminthosporin and catenarin.

FIGURE 12. *Penicillium islandicum* Sopp N.R.R.L. 1036 (magn.  $\times 250$ ). Showing crystals of colouring matters in mycelium. Source of islandicin.

## PLATE 26

FIGURE 13. *Penicillioopsis clavariaeformis* Solms-Laubach. Showing typical growth in 11. flask on a synthetic medium fortified with orange juice. Source of penicilliopsin.

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# A class of enumerations of importance in genetics

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A number of enumerations arising in genetics, e.g. of sets of isomorphic genotypes, of modes of gamete formation, of the modes of formation of ordered and unordered tetrads, etc., have been found in previous work to yield solutions expressible as the mean powers of arbitrary degree of appropriate series of bases. These are such as to yield integral values of the mean for all integral exponents.

The general combinatorial theorem governing this class of enumeration is given in § 2. Other sections illustrate the method in operation by confirming and extending the genetic formulae so far established.

In § 3.1 is a brief discussion of a partitional function which serves to enumerate the numbers of partitions of any partible number in a lattice of arbitrary dimensionality.

## 1. ILLUSTRATIVE EXAMPLES

During recent years a number of the problems encountered in genetic analysis have been found to involve problems of enumeration, the solutions of which were far from obvious, although structurally similar.

Among the first of these to have the solution published (Fisher 1947) were (i) the number of modes of gamete formation in tetrasomic organisms with  $l$  linked loci, for which the formula

$$\frac{1}{48} \cdot 16^l + \frac{1}{3} \cdot 4^l + \frac{1}{3},$$
$$2, \quad 11, \quad 107, \quad 1451, \quad \dots,$$

was demonstrated by a somewhat indirect route. In the same paper the formula (ii)

$$\frac{1}{6!3!} (210^l + 15 \cdot 60^l + 3 \cdot 30^l + 40 \cdot 24^l + 45 \cdot 20^l + 135 \cdot 10^l + 335 \cdot 6^l + 320 \cdot 3^l + 630 \cdot 2^l),$$
$$2, \quad 40, \quad 3175, \quad \dots,$$

was offered tentatively for hexasomic forms. This has since been verified by the general method to be here discussed. In an appendix also was given without proof the enumeration (iii)

$$\frac{1}{24} (14^l + 9 \cdot 6^l + 14 \cdot 2^l),$$
$$4, \quad 24, \quad 200, \quad 2096, \quad \dots,$$

for the number of sets of isomorphic genotypes of a tetrasomic organism heterogenic at  $l$  loci. No attempt was made at that time to enumerate the numbers for a hexasomic organism, for which, when  $l$  is unity, the number is 10, and when  $l = 2$ , the number of sets was found to be 277. It will later be shown that the number using  $l$  loci is in general

$$\frac{1}{6!} (202^l + 15 \cdot 66^l + 60 \cdot 30^l + 40 \cdot 19^l + 120 \cdot 9^l + 180 \cdot 8^l + 40 \cdot 7^l + 120 \cdot 3^l + 144 \cdot 2^l),$$
$$10, \quad 277, \quad 20343, \quad \dots,$$

Apart from their genetical meaning, the two examples last given are in general combinatorial theory to be recognized as the numbers of partitions of the partible numbers 4 and 6 in  $l$  dimensions.

Since the events of a single reduction division are capable of determining not a single gamete only, but a tetrad of two pairs of gametes, the joint content of which specifies more completely than a single gamete the course of the preceding events, it is relevant also to inquire as to the number of modes of tetrad formation. For disomic organisms this is shown without great difficulty to be

$$\frac{1}{16}(6^l + 3 \cdot 4^l + 7 \cdot 2^l),$$

$$2, \quad 7, \quad 29, \quad 136, \quad 692, \quad 3712, \quad \dots$$

With tetrasomic or hexasomic organisms the problem is much more complex. The same is true of the specification of *matings*; e.g. those involving two disomic genotypes, supposing them to be not equivalent homozygotes at any of the loci concerned, or, in other words, ignoring all loci at which the mating is homogenic; then the number of mating types heterogenic at exactly  $l$  loci is found to be

$$\frac{1}{8}(14^l + 5 \cdot 6^l + 2 \cdot 2^l),$$

$$6, \quad 48, \quad 480, \quad 5616, \quad \dots,$$

The question might be asked equally of polysomic organisms, or in respect of two or more matings.

## 2. COMBINATORIAL STRUCTURE COMMON TO THIS CLASS OF PROBLEM

Let us suppose that a formula is to be constructed by inserting appropriate 'contents' into a finite number of cells or 'containers'. Inherent in the data of each problem is a certain finite group of permutations among the containers, or among the elements of the contents. If any formula can be derived from another by a permutation of this basic group, the two formulae are said to belong to the same set; what is to be enumerated is the number of such sets.

It will be noticed that the formulae derived from any given formula by permutations of the basic group, by which the sets are defined, may be all different, in which case the set will contain a number of formulae equal to the order  $N$  of the basic group, or alternatively that a subgroup of this group leaves the formula unaltered, and that in this case the number of different formulae in the set is reduced by a factor which is the order of the invariant subgroup.

If, starting with one member of each set, we apply all permutations of the group, including the identity, we shall develop an assemblage of formulae numbering in all  $N$  times as many as the number of sets. In this assemblage every possible formula must occur, and the frequency of its occurrence is equal to the order of the subgroup by which it is unaltered.

Now the sum for all formulae of the orders of the subgroups of permutations by which they are unaltered is equal to the sum for all permutations of the numbers of formulae unaltered by them. Suppose, now, for any given permutation there are  $a$  ways of inserting contents which shall be unaltered by this permutation, then, if the

contents are added in  $l$  successive layers, there must be  $a^l$  invariant compound formulae possible. The number of sets is then given by

$$\frac{1}{N} S(a^l),$$

where  $S$  stands for summation over all the  $N$  permutations, including the identity, of the given group.

### 3. OPERATIONAL PROCEDURE; ISOMORPHIC SETS OF GENOTYPES

Consider as a first illustration the number of isomorphic sets of tetrasomic genotypes.

There are four chromosomes, and the complete permutation group of these four generates members of the same set. The twenty-four elements of this group may be classified according to the partitions of 4, as follows:

1	without change, the identity	(1 <sup>4</sup> )
6	interchanges of pairs	(21 <sup>2</sup> )
3	double interchanges of pairs	(2 <sup>2</sup> )
8	cycles of three	(31)
6	cycles of four	(4)
<hr/>		
24	total	

The same partitions also can be used to specify the cell contents at any one locus; i.e. (1<sup>4</sup>) stands for the case of four different allelomorphous genes, (21<sup>2</sup>) for the case where two only are alike, (2<sup>2</sup>) for the case where there are two pairs of like genes, and (31) for the case of three alike and one different. The last case (4), representing four genes all alike, will be omitted, since the enumeration is to be in terms of the number of heterogenic loci, so that no homogenic locus need be considered.

The number of formulae of these kinds, equal to the number invariant with the identity, and the number invariant with the other classes of possible permutations, are set out in table 1.

TABLE 1. NUMBER OF DISTRIBUTION FORMULAE OF DIFFERENT PARTITIONAL TYPES INVARIANT FOR DIFFERENT TYPES OF PARTITION

distribution formulae		type of permutation					total 24
partition	number	6 (4)	8 (31)	3 (2 <sup>2</sup> )	6 (21 <sup>2</sup> )	1 (1 <sup>4</sup> )	
(31)	4	—	1	—	2	4	
(2 <sup>2</sup> )	3	1	—	3	1	3	
(21 <sup>2</sup> )	6	—	—	2	2	6	
(1 <sup>4</sup> )	1	1	1	1	1	1	
total		2	2	6	6	14	

The number of distribution formulae for a given partition

$$P = (p_1^{\pi_1} p_2^{\pi_2} \dots), \quad (p\pi) = n,$$

is

$$\frac{n!}{(p_1!)^{\pi_1} \dots (\pi_1!) \dots},$$

appropriately to the fact that the interchange of any two equally numerous genes is recognized to be a matter of indifference; the numbers of permutations of different partitional types are, however,

$$\frac{n!}{p_1^{\pi_1} \dots (\pi_1!) \dots};$$

these add to  $n!$  Each is a permutation in  $\pi_1$  cycles of  $p_1$ ,  $\pi_2$  cycles of  $p_2$ , etc.

The individual entries in the body of table 1 show for each of the five types of permutation for how many distribution formulae (of four different types in the four rows) each is inoperative. The sums of the five columns show that

For 1 permutation (the identity) there are 14 invariant formulae,

For 9 permutations there are 6 invariant formulae,

For 14 permutations there are 2 invariant formulae.

Since the permutation group of four objects is of order 24, the enumeration formula is now obtained as

$$\frac{1}{24}(14^l + 9 \cdot 6^l + 14 \cdot 2^l),$$

giving the number of partitions in (exactly)  $l$  dimensions of the partible number 4, or, the number of sets of isomorphic genotypes in a tetrasomic organism heterogenic at (exactly)  $l$  loci.

For partitions of 6, or for hexasomic genotypes, we have, instead of table 1, a similar table of ten rows and eleven columns, shown below as table 2. giving, as previously stated, the enumeration

$$\frac{1}{720}(202^l + 15 \cdot 66^l + 60 \cdot 30^l + 40 \cdot 19^l + 120 \cdot 9^l + 180 \cdot 8^l + 40 \cdot 7^l + 120 \cdot 3^l + 144 \cdot 2^l).$$

$$10, \quad 277, \quad 20343, \quad \dots,$$

In constructing the table it is a convenient check that the products of the entries in each row with the numbers at the head of the columns add in each case to 720.

The table for partitions of 8 is shown in table 3. The work is heavier here, though most of the entries can be written down at sight. The enumeration formula is, however, not very lengthy, as it has only 19 terms. It may be written in the form

$$\begin{aligned} & \frac{1}{40320} \cdot 4139^l + \frac{1}{1440} \cdot 1079^l + \frac{1}{192} \cdot 351^l + \frac{1}{360} \cdot 254^l + \frac{5}{384} \cdot 163^l + \frac{1}{36} \cdot 86^l \\ & + \frac{1}{96} \cdot 81^l + \frac{1}{24} \cdot 42^l + \frac{3}{32} \cdot 37^l + \frac{1}{36} \cdot 32^l + \frac{1}{36} \cdot 20^l + \frac{1}{30} \cdot 19^l + \frac{1}{32} \cdot 15^l \\ & + \frac{1}{12} \cdot 12^l + \frac{1}{6} \cdot 10^l + \frac{1}{10} \cdot 9^l + \frac{1}{15} \cdot 4^l + \frac{1}{8} \cdot 3^l + \frac{1}{7} \cdot 2^l. \end{aligned}$$

The numerical values are

$$21, \quad 2974, \quad 2991002, \quad \dots$$

### 3.1. *Properties of a special partitional function*

In connexion with this mode of enumerating the number of partitions of any partible number in  $l$  dimensions, it may be noted that the general result may be expressed in terms of two partitional functions.

If  $P$  stands for any partition of the partible number  $n$

$$P = (p_1^{\pi_1} p_2^{\pi_2} \dots), \quad \Sigma(p\pi) = n,$$

TABLE 2. PARTITIONS OF 6

partition type for single locus	types and numbers of chromosome permutations														720
	( <i>abcdef</i> ) 120	( <i>abcde</i> ) 144	( <i>abcd</i> ) ( <i>ef</i> ) 90	( <i>abcd</i> ) 90	( <i>abc</i> ) ( <i>def</i> ) 40	( <i>abc</i> ) ( <i>de</i> ) 120	( <i>abc</i> ) 40	( <i>ab</i> ) ( <i>cd</i> ) ( <i>ef</i> ) 15	( <i>ab</i> ) ( <i>cd</i> ) 45	( <i>ab</i> ) 15	( <i>ab</i> ) 15	( <i>ab</i> ) 15	( <i>ab</i> ) 15	( <i>ab</i> ) 15	
(51)	.	1		2		1	3	.	2	4	6				
(42)	.	.	1	1	.	1	3	3	3	7	15				
(3 <sup>2</sup> )	1	.	2	1	1	1	1	4	2	4	10				
(41 <sup>2</sup> )	.	.	1	1	.	1	3	3	3	7	15				
(321)	.	.	.	1	.	1	3	3	4	16	60				
(2 <sup>3</sup> )	1	.	1	1	3	.	.	7	3	3	15				
(31 <sup>4</sup> )	.	.	.	.	2	2	2	.	4	8	20				
(2 <sup>2</sup> 1 <sup>2</sup> )	.	.	1	1	.	.	.	9	5	9	45				
(21 <sup>4</sup> )	.	.	1	1	.	1	3	3	3	7	15				
(1 <sup>6</sup> )	1	1	1	1	1	1	1	1	1	1	1				
total	3	2	8	8	7	9	19	30	30	66	202				

TABLE 3. PARTITIONS OF 8

	(8)	(71)	(62)	(53)	(42)	(612)	(521)	(431)	(422)	(322)	(512)	(4212)	(3212)	(3221)	(24)	(414)	(3212)	(3212)	(312)	(2214)	(214)	(13)
	5040	5760	3360	2688	1260	3360	4032	3360	1260	1120	1344	2520	1120	1680	105	420	1120	420	112	210	28	1
(71)	8																					
(62)	28	1	1	1	1	2	1	1	1	1	3	2	2	1	1	4	3	2	5	4	6	8
(53)	56									2	3	2	1	2	4	6	4	4	10	8	16	28
(43)	35				3						1	2	2	3		4	5	6	11	12	26	56
(612)	28		2					1	5			1	2	1	11	1	3	3	5	7	15	35
(521)	168		1			1	1		2	1	3	2	1	2	4	6	4	4	10	8	16	28
(431)	280						1				3	2		2		12	6	6	30	20	66	168
(422)	210				2			1	4			2	4	1	18	4	9	6	25	28	90	280
(322)	280		1			1			4	1		2	1	3	16	4	3	12	15	18	60	210
(512)	56			1			1	1		2	1	2	2	3			4	10	10	20	70	280
(4212)	420								2			2		2	12	6	6	12	30	32	120	420
(3212)	280		1			1			4	1		2	1	2	16		4	10	10	20	70	280
(3221)	840							1				2	2	3		4	3	18	15	36	150	840
(24)	105	1	1		5	1			3	3		1	3		25	3		7		9	15	105
(414)	70			2			2	2	2			2	4	2	6	2	6	6	10	14	30	70
(3212)	560									2			2	4			8	12	20	40	140	560
(2312)	420		1			1			2	3		2	3		28			16		24	60	420
(315)	56			1			1	1		2	1	2	2	3		4	5	6	11	12	26	56
(2214)	210				2		1	1	4			2			18	4	3	12	15	18	60	210
(216)	28		1			1	1		2	1	3	2	1	2	4	6	4	4	10	8	16	28
(18)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	3	2	10	4	15	10	9	12	37	20	19	37	32	42	163	81	86	163	254	351	1079	4139

and  $\alpha(P)$  stands for the partitional function

$$\alpha(P) = \frac{n!}{p_1^{r_1} \dots \pi_1! \dots},$$

then  $\alpha(P)$  enumerates the numbers of permutations of given partitional type.

The method of § 3 has also defined a second partitional function, which we may denote by  $e(P)$ , such that the number of partitions of  $n$  in  $l$  dimensions is

$$\sum_{P/n} (e-1)^l \alpha / \sum_{P/n} \alpha,$$

in which the summation is taken over all partitions in 0 or 1 dimension of the number  $n$ , so that  $\Sigma(\alpha) = n!$ . The number of partitions in 0 or 1 dimension is therefore

$$1 + \frac{\Sigma(e-1)\alpha}{\Sigma(\alpha)} = \Sigma(e\alpha)/\Sigma(\alpha),$$

or the average value of  $e$  over all permutations.

For small partitions the numerical value of  $e$  may be obtained more expeditiously than by the construction of the bipartitional table, for when  $P = (1^n)$  it is seen that  $e$  is the coefficient of  $x^n/n!$  in the expansion in powers of  $x$  of

$$F(x) = e^{e^x-1}.$$

The series of coefficients is

$$1, \quad 1, \quad 2, \quad 5, \quad 15, \quad 52, \quad 203, \quad 877, \quad 4140,$$

up to the eighth degree.

Given the contents of any partition we may build up a differential operator competent to give the partitional function  $e$ , by acting upon  $F(x)$ . For unit parts we need only insert a factor  $D$  for each part; for prime parts a factor  $(D+1)$ ; for compound parts having  $f$  factors (excluding unity), the factor is  $(D+f)$ . If, then, no two parts have a common factor, the differential operator is complete. Thus for the partition  $(321^3)$  we have the operator

$$D^3(D+1)^2$$

yielding

$$52 + 2(15) + 5 = 87,$$

in accordance with the entry [giving  $(e-1)$ ] in table 3.

When some of the parts have common factors we have to introduce also the sums of the factors common to any two parts, the sums of the squares of the factors common to any three, and so on. So for the partition  $P = (6^2)$ , we find

$$(D+3)^2 + 11 = D^2 + 6D + 20,$$

and for  $P = (2^3)$  the operator is

$$(D+1)^3 + 3.2.(D+1) + 4 = D^3 + 3D^2 + 9D + 11,$$

giving

$$e(P) = 5 + 6 + 9 + 11 = 31,$$

in accordance with the entry in table 2.

4. MODES OF GAMETE FORMATION

Consider a tetrasomic organism having (of any particular kind) four homologous chromosomes,  $A, B, C$  and  $D$ . The gametes will be diploid, and will contain for each locus two representative genes. A gamete may then be represented by

$$\begin{aligned} AA & \text{ 4 formulae,} \\ AB & \text{ 12 formulae.} \end{aligned}$$

Gametes of the first kind are said to exhibit double reduction at the locus in question. However many loci are considered, the gamete represented by any formula will be said to have originated by the same mode of gamete formation as that represented by any formula derivable from the first:

(a) by reversal of the symbols for the gametic chromosomes, an operation which may be represented by the symbol  $r$ ,

(b) by permutation of the parental chromosomes, represented by such symbols as  $(ab)$ ,  $(abc)$ ,  $(ab)(cd)$ , etc., 24 in all, including the identity  $I$ .

It is now easy to consider for each of the 48 possible operations, how many of the 16 formulae specified above are invariant.

		permutations
for the identity	all 16 formulae are invariant	1
for $r$	4 formulae $AA, BB, CC, DD$	1
$(ab)$	4 formulae $CC, DD, CD, DC$	6
$(ab) r$	4 formulae $CC, DD, AB, BA$	6
$(ab)(cd)$	0	3
$(ab)(cd) r$	4 formulae $AB, BA, CD, DC$	3
$(abc)$	1 formula $DD$	8
$(abc) r$	1 formula $DD$	8
$(abcd)$	0	6
$(abcd) r$	0	6
		<hr/> 48

The formula enumerating the modes of gamete formation is therefore

$$\frac{1}{48}(16^l + 16 \cdot 4^l + 16),$$

or, more simply,

$$3a_l^2 - 1,$$

where

$$a_l = \frac{1}{12}(4^l + 8)$$

is easily calculated, since

$$a_l = 4a_{l-1} - 2$$

$l$	1	2	3	4	5	6
$a$	1	2	6	22	86	342
$3a^2 - 1$	2	11	107	1451	22187	350891 ...

By the methods previously employed the enumeration of the modes of gamete formation in hexasomic organisms presented a very intricate problem, and I had little confidence in the formula given. The method of this paper gives the same result by a much simpler path. The two modes of formation for a single locus are easily seen to give the two types of formula:

type	number of formulae
$ABC$	120
$AAB$	90



The number of these is to be ascertained for a group of permutations involving changes of two kinds. (i) Permutations of the position in which the three elements of the gamete are written down. These are six in number, being the identity (*I*), three interchanges of pairs (12), (23), (31), and two cyclic changes (123), (132). (ii) There are the 6! permutations of the six letters *a, b, c, d, e* and *f*, standing for the parental chromosomes. The whole group of 6! 3! changes is exhibited in a two-way table, in which the number of invariant members of each class of formula have been entered separately.

TABLE 4. NUMBERS OF GAMETIC FORMULAE INVARIANT FOR VARIOUS PERMUTATIONS

permutations of letters (parental chromosomes)	permutations of order (gametic chromosomes)			number type
	1 <i>I</i>	3 (12)	2 (123)	
1 <i>I</i>	120, 90	0, 30	0, 0	
15 ( <i>ab</i> )	24, 36	8, 12	0, 0	
45 ( <i>ab</i> ) ( <i>cd</i> )	0, 6	8, 2	0, 0	
40 ( <i>abc</i> )	6, 18	0, 6	3, 0	
15 ( <i>ab</i> ) ( <i>cd</i> ) ( <i>ef</i> )	0, 0	0, 0	0, 0	
120 ( <i>abc</i> ) ( <i>de</i> )	0, 0	2, 0	3, 0	
90 ( <i>abcd</i> )	0, 6	0, 2	0, 0	
40 ( <i>abc</i> ) ( <i>def</i> )	0, 0	0, 0	6, 0	
90 ( <i>abcd</i> ) ( <i>ef</i> )	0, 0	0, 0	0, 0	
144 ( <i>abcde</i> )	0, 0	0, 0	0, 0	
120 ( <i>abcdef</i> )	0, 0	0, 0	0, 0	

Adding the numbers for the two types of formulae, it appears that

1 permutation is inoperative for 210 formulae  
 15 permutations are inoperative for 60 formulae  
 3 permutations are inoperative for 30 formulae  
 40 permutations are inoperative for 24 formulae  
 45 permutations are inoperative for 20 formulae  
 135 permutations are inoperative for 10 formulae  
 335 permutations are inoperative for 6 formulae  
 320 permutations are inoperative for 3 formulae  
 630 permutations are inoperative for 2 formulae

giving the formula stated above, equivalent to

$$\frac{1}{4320}210^l + \frac{1}{388}60^l + \frac{1}{1440}30^l + \frac{1}{1080}24^l + \frac{1}{96}20^l + \frac{1}{32}10^l + \frac{67}{864}6^l + \frac{2}{27}3^l + \frac{7}{48}2^l.$$

2, 40, 3175, ....

Tetrasomic gametes from octosomic parents may be of three kinds

*ABCD* with 1680 formulae  
*AABC* with 2016 formulae  
*AABB* with 168 formulae

Table 5 shows in columns corresponding with permutations of the gametic chromosomes, and rows with permutations of the parental chromosomes, the number of formulae invariant to each type. Since no formula is unaltered by some of the permutations of the parental chromosomes, these have been omitted.

TABLE 5. NUMBERS OF GAMETIC FORMULAE INVARIANT FOR VARIOUS PERMUTATIONS SEGREGATING IN OCTOSOMICS

		type of permutation of gametic chromosome				
		1	6	3	8	6
		<i>I</i>	(12)	(12) (34)	(123)	(1234)
1	<i>I</i>	3864	392	56	.	.
28	( <i>ab</i> )	1170	222	58	.	2
210	( <i>ab</i> ) ( <i>cd</i> )	204	100	60	.	4
112	( <i>abc</i> )	540	80	20	15	.
420	( <i>ab</i> ) ( <i>cd</i> ) ( <i>ef</i> )	6	26	62	.	6
1120	( <i>abc</i> ) ( <i>de</i> )	54	30	22	9	2
420	( <i>abcd</i> )	204	36	12	.	4
105	( <i>ab</i> ) ( <i>cd</i> ) ( <i>ef</i> ) ( <i>gh</i> )	.	.	64	.	8
1680	( <i>abc</i> ) ( <i>de</i> ) ( <i>fg</i> )	.	4	24	3	4
1120	( <i>abc</i> ) ( <i>def</i> )	6	2	2	12	.
2520	( <i>abcd</i> ) ( <i>ef</i> )	6	10	14	.	6
1344	( <i>abcde</i> )	54	12	6	.	.
1120	( <i>abc</i> ) ( <i>def</i> ) ( <i>gh</i> )	.	.	4	.	2
1260	( <i>abcd</i> ) ( <i>ef</i> ) ( <i>gh</i> )	.	.	16	.	8
3360	( <i>abcd</i> ) ( <i>efg</i> )	.	.	.	3	4
4032	( <i>abcde</i> ) ( <i>fg</i> )	.	2	8	.	2
3360	( <i>abcdef</i> )	6	2	2	.	.
1260	( <i>abcd</i> ) ( <i>efgh</i> )	.	.	.	.	8
3360	( <i>abcdef</i> ) ( <i>gh</i> )	.	.	4	.	2

The enumerating function, in 31 terms, is then:

3864 <sup><i>l</i></sup>			
28.1170 <sup><i>l</i></sup>	1260.62 <sup><i>l</i></sup>	5040.24 <sup><i>l</i></sup>	15120.10 <sup><i>l</i></sup>
112.540 <sup><i>l</i></sup>	630.60 <sup><i>l</i></sup>	3360.22 <sup><i>l</i></sup>	8960.9 <sup><i>l</i></sup>
6.392 <sup><i>l</i></sup>	84.58 <sup><i>l</i></sup>	336.20 <sup><i>l</i></sup>	27846.8 <sup><i>l</i></sup>
168.222 <sup><i>l</i></sup>	3.56 <sup><i>l</i></sup>	3780.16 <sup><i>l</i></sup>	29092.6 <sup><i>l</i></sup>
630.204 <sup><i>l</i></sup>	2464.54 <sup><i>l</i></sup>	896.15 <sup><i>l</i></sup>	57540.4 <sup><i>l</i></sup>
1260.100 <sup><i>l</i></sup>	2520.36 <sup><i>l</i></sup>	7560.14 <sup><i>l</i></sup>	40320.3 <sup><i>l</i></sup>
672.80 <sup><i>l</i></sup>	6720.30 <sup><i>l</i></sup>	18284.12 <sup><i>l</i></sup>	122472.2 <sup><i>l</i></sup>
315.64 <sup><i>l</i></sup>	2520.26 <sup><i>l</i></sup>		

all divided by 8!4!, or 967680.

The numerical values, when *l* is 1, 2 and 3, are 3, 188 and 135188.

5. TETRAD FORMATION

Another case in which the number of formulae is not so great that they cannot easily be examined individually is that of tetrad formation in diploid organisms. If all four products of the same meiosis can be observed, and if the pair of products of the first division can be distinguished, we have an ordered tetrad, with formulae of two kinds:

type of formula	number
<i>A, A; a, a</i>	2
<i>A, a; A, a</i>	4

The permutation group by which representations of the same mode of tetrad formation can be recognized consists of (i) two interchanges within the pairs of

products of the second division, which may be represented by the symbols  $p, q$ ; (ii) interchange between the products of the first division, represented by  $r$ ; (iii) interchange of the gene symbols ( $Aa$ ). The group is of order 16.

Then it is easy to verify table 6, showing the number of formulae invariant for any combination of these operations.

TABLE 6. NUMBER OF FORMULAE FOR ORDERED TETRAIDS INVARIANT FOR DIFFERENT INTERCHANGES

reversals of position	gene interchange	
	$I$	$(Aa)$
1 $I$	6	—
2 $p, q$	2	—
1 $pq$	2	4
1 $r$	2	4
2 $rp, rq$	—	2
1 $rpq$	2	4

The number of modes of formation of ordered tetrads is therefore

$$\frac{1}{16}(6^l + 3 \cdot 4^l + 7 \cdot 2^l),$$

$$2, \quad 7, \quad 29, \quad 136, \quad 692, \quad \dots$$

If the products of the second division are not recognizable, we shall have an unordered tetrad. Here there is only one type of formula

$$AAaa,$$

which can be written in 6 ways. Permutation among these four elements, in 24 ways, together with interchange of the gene symbols gives a group of order 48, with invariance shown by table 7.

TABLE 7. NUMBERS OF FORMULAE FOR UNORDERED TETRAIDS INVARIANT FOR DIFFERENT PERMUTATIONS

permutations of position	gene interchange	
	$I$	$(Aa)$
1 $I$	6	—
6 $(ab)$	2	—
3 $(ab)(cd)$	2	4
6 $(abcd)$	—	2

The number of modes of formation for unordered tetrads is therefore

$$\frac{1}{48}(6^l + 3 \cdot 4^l + 15 \cdot 2^l),$$

$$1, \quad 3, \quad 11, \quad 48, \quad 236, \quad \dots$$

With ordered tetrads for tetrasomic organisms, the basic group contains four additional interchanges between the chromosomes of the four diploid gametes, while the gene interchange is replaced by a group of 24 permutations of the parental chromosomes. The order of the group is thus  $2^7 \cdot 4!$ .

I find this case very confusing, and have not thoroughly checked the result. It is put on record as an aid to any later worker who may consider the problem:

$$\frac{4^l}{27 \cdot 4!} (630^l + 4 \cdot 90^l + 51 \cdot 24^l + 10 \cdot 18^l + 102 \cdot 12^l + 161 \cdot 6^l + 252 \cdot 4^l + 24 \cdot 3^l + 84 \cdot 2^l + 352)$$

8, 2538, ....

The unordered tetrads will be about one-third as numerous.

#### REFERENCE

- Fisher, R. A. 1947 The theory of linkage in polysomic inheritance. *Phil. Trans. B*, **233**, 55-87.

## The utilization of carbon sources by *Bact. lactis aerogenes*

### I. General survey

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A survey is made of the ability of various carbon compounds to support growth of *Bact. lactis aerogenes*. They are classified into those to which the cells (a) cannot become adapted, (b) become adapted by training, (c) are fully adapted on transfer from a glucose medium in which they are normally grown.

With class (b) there is usually a long initial lag on the first transfer and an initial growth rate well below the optimum.

Phenomena of 'cross-adaptation' appear, training to an earlier member of the series acetic acid, succinic acid, fumaric acid entailing adaptation to a later member but not vice versa.

With citric acid, aconitic acid and glycerol there is evidence of multiple-growth mechanisms.

Additions of certain carboxylic acids shorten the lag of ageing cultures in the glucose medium.

The classes (a), (b) and (c) bear no relation to the number of carbon atoms or the functional groups in the molecules of the various substances. They bear, however, certain relations to the 'tricarboxylic acid cycle', which are examined in part II.

#### INTRODUCTION

The experiments to be described form part of a systematic study of the internal economy of the coliform organism *Bact. lactis aerogenes*, and deal with certain aspects of the mechanism by which carbon compounds are utilized for aerobic growth.

The cells are unexacting, readily take their nitrogen from ammonium salts, and will grow on a variety of different carbon compounds, including glycerol, glucose and many other carbohydrates. Complex added growth factors are not required, although in a simple synthetic medium consisting of ammonium sulphate, glucose,

inorganic ions and phosphate buffer, there may be a lag phase during which a diffusible intermediate, necessary for multiplication, escapes into the medium (Lodge & Hinshelwood 1943). Towards the end of the lag phase, moreover, traces of glutamic and aspartic acids appear in the solution (Morrison & Hinshelwood 1949).

When cells are transferred from glucose media to other carbon sources a period of training and adaptation is not infrequently necessary, the precise pattern of the adaptive phenomena varying from one example to another (for references see Hinshelwood 1946).

It is evident that the cells may achieve their final synthetic ends by various networks of reactions, and that the mechanisms of all the possible parts of these networks are not always fully mobilized. During lag phases necessary intermediates have to be built up in the appropriate combinations, and during adaptive processes slow growth occurs with modification of the actual enzyme balance of the cell.

With the object of discovering more about the structural relationships of the carbon compounds which *Bact. lactis aerogenes* is able to handle, we set ourselves originally the problem of inquiring into the probable significance for this organism of, among other compounds, those occurring in the well-known 'tricarboxylic acid cycle'. This scheme has been proposed as a mechanism for the oxidation of pyruvic acid in various biological processes (Szent-Györgyi 1935, 1936; Krebs 1943). It has proved expedient to examine a rather wider range of compounds than those included in the scheme, and the plan has been to examine the following questions:

(1) Which of a considerable number of fairly simple carbon compounds are in fact utilizable by the cells of *Bact. lactis aerogenes*. (The majority of those occurring in the tricarboxylic acid cycle are included, and in part II the question will be considered whether these are preferred to others of somewhat similar carbon content and structure.)

(2) Whether the utilizable substances will support growth immediately or only after the cells have become adapted. (This will later allow us to inquire whether there is any presumption that normal growth, in glucose, involves the specific mechanisms of the tricarboxylic acid cycle in a fully mobilized condition.)

(3) Whether any adaptive processes which occur may prove to be common to more than one of the substances studied.

(4) Whether additions to normal growth media of specific substances influence the duration of the lag phase, and what relation exists between efficiency in this respect and power to support growth independently.

The answers to these questions should yield evidence about the mode of linking of the cell reactions in this particular organism. In part II the significance of the tricarboxylic acid cycle will be specifically dealt with.

#### EXPERIMENTAL METHODS

The general experimental methods were as described in previous papers from this laboratory (Lodge & Hinshelwood 1943; Hinshelwood & Lodge 1944; Postgate & Hinshelwood 1946).

The growth media, which were aerated, consisted of phosphate buffer, magnesium sulphate, ammonium sulphate and the carbon compound under examination. Other necessary details will be recorded in the relevant places.

The initial pH was always adjusted to 7.12.

#### TYPES OF BEHAVIOUR WITH DIFFERENT CARBON SOURCES

Table 1 includes all compounds studied as sole carbon substrate for this strain of *Bact. lactis aerogenes* with the exception of certain disaccharides and polysaccharides.

In it the substances are classified according to their ability to support growth.

Substances which fail to support growth call for little comment. Oxalic acid, propionic acid and tartronic acid supported no growth within 4 to 5 days of inoculation. Of these substances only propionic acid appears to inhibit growth in the glucose-ammonium sulphate medium. Tartronic acid and formic acid do not increase the length of the lag phase in this (the usual standard) medium. With a certain culture it was 23 hr. in the latter and with the two additions less than 20 and 23 hr. respectively. In a similar experiment the lag phases were in the ratios: glucose 1.0; with added formic acid 1.0; with added tartronic acid 1.03.

TABLE 1. CLASSIFICATION OF COMPOUNDS

compounds towards which the cells are already fully adapted on transfer from a glucose medium	compounds towards which the cells undergo adaptation	compounds failing to support growth
glyceric acid (?)	acetic acid	formic acid
pyruvic acid	glycerol	oxalic acid
lactic acid	malonic acid	propionic acid
D L-malic acid	succinic acid	tartronic acid
L-arabinose	fumaric acid	erythritol
D-xylose	maleic acid	L-tartaric acid
glucose	D-tartaric acid	
inositol	meso-tartaric acid	
citric acid	glutaric acid	
cis-aconitic acid	$\alpha$ -keto-glutaric acid	
	D-arabinose	
	adipic acid	

A slightly more elaborate way of testing whether the substrate is utilized is illustrated by the following experiment. Serial subcultures were made into media containing sodium formate at 3000 mg./l. together with diminishing amounts of glucose. The final total bacterial populations were recorded (table 2).

TABLE 2. SUBCULTURES IN GLUCOSE-FORMATE MIXTURES

number of subculture	glucose (mg./l.)	formate (mg./l.)	total bacterial population (millions/ml.)
1	400	3000	155
2	200	3000	75
3	100	3000	47
4	100	0	50
5	0	3000	0

It is obvious that the sodium formate has no appreciable effect upon the final population which the medium can support.

With substances towards which the cells are not already fully adapted the behaviour conforms to one of two types: (a) A long initial lag ( $L$ ) is followed by slow growth (mean generation time,  $T$ ). Both  $T$  and  $L$  shorten on serial subculture. (b)  $L$  itself is short but is followed by slow growth, the rate of which increases during the course of training by subculture.

The former type of behaviour is shown with acetic acid, succinic acid and fumaric acid, the latter with glycerol, and possibly to a slight extent with D-xylose and L-arabinose (Cooke & Hinshelwood 1947). No other representative of this class occurs amongst the compounds in table 1. All the others which show adaptation belong to class (a). No instance has yet been found where  $L$ , for cultures of optimal age, retains a high value after about five or six subcultures.

Table 3 gives some examples of the shortening of  $L$  on serial subculture. The values given are for 0.1 ml. inocula withdrawn from actively growing cultures. *Bact. lactis aerogenes* would give values between 0 and 2 hr. under such conditions in glucose-ammonium sulphate cultures.

TABLE 3. TYPICAL VALUES FOR THE DURATION OF THE LAG PHASE SHOWING ITS SHORTENING AS THE CELLS BECOME ADAPTED

carbon source	$L$ (min.)		
	on first subculture	on second subculture	After more than ten subcultures
acetic acid	1560	720	30
	840	480	30
	660	330	30
	480	240	150
succinic acid	5400	510	60
	3000	300	0
	1020	200	—
	600	—	—
fumaric acid	350	60	30
malonic acid	1800	—	60
	900	200	—
maleic acid	500	—	60
glutaric acid	1800	—	60
adipic acid	3600	200	60
D-tartaric acid	600 to 1200	200	60
meso-tartaric acid	1500	—	60
$\alpha$ -keto-glutaric acid	500	—	60
D-arabinose	1800	200	30

Some illustrations of behaviour of the type (a) are shown in figures 1 and 2.

The lag phase in these examples is usually not completely removed by one subculture. Even after five or six 'training' subcultures it is occasionally still appreciable, particularly if the cells have been allowed to age for some time in a fully grown culture. For instance, after five training subcultures in a succinic acid medium the cells showed a lag of 15 hr. in that medium and only  $2\frac{1}{2}$  hr. in the glucose medium. Thus  $L$  increases far more rapidly with age if the cells are subcultured in the medium

towards which they are being trained than if subcultured into the glucose medium. Addition of glucose to the medium will sometimes cause growth, in a few hours, of

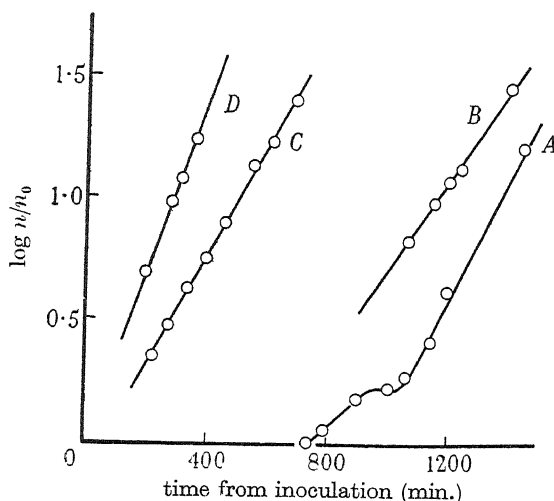


FIGURE 1. Training to acetic acid as carbon source. *A*, 1st subculture (strain 1); *B*, 1st subculture (strain 2); *C*, 9th subculture. *D*, 60th subculture.  $n_0$  = initial bacterial count;  $n$  = count at time shown.

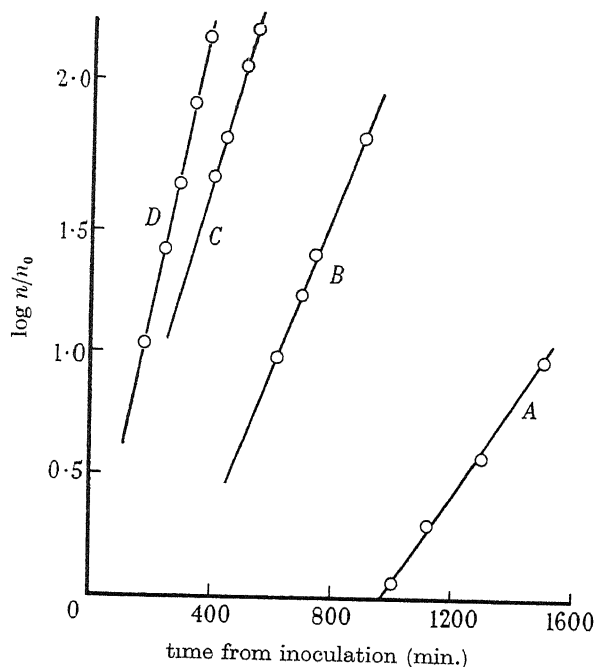


FIGURE 2. Training to succinic acid as carbon source. *A*, 1st subculture; *B*, 2nd subculture; *C*, 10th subculture; *D*, 45th subculture.

cells which would otherwise have died. This occurs even with fully adapted strains in acetic acid and succinic acid media, where the addition of glucose will revive apparently dying cultures.



The variation of  $L$  with the age of a parent culture growing for the first time in an acetate medium was determined for transfers both to more acetate and to glucose. The minimum value of  $L$  was 4 hr. in acetic acid and  $2\frac{1}{2}$  hr. in glucose. Since, however, when training is complete, the minimum value of  $L$  approached zero for subculture into glucose, this  $2\frac{1}{2}$  hr. does not represent a loss of glucose adaptation so much as a temporary derangement of the enzyme balance when the cell first begins to grow upon a new substrate.

Some other typical values for  $L$  on subculture from actively growing cultures of the training medium into a fresh supply of the same, on the one hand, and into glucose on the other, are given in table 4, which shows how the lag at the end of the adaptation process has dropped to a small value both in the training medium and in the glucose medium.

TABLE 4. LAGS DURING TRAINING

substrate	number of previous subcultures in training medium	lag in training medium (min.)	lag in glucose medium (min.)
acetic acid	1	240	150
	6	150	480
	19	0	90
	1	324	344
succinic acid	1	300	300
	1	510	240
	1	338	300
	1*	100	100
	19	0	50
	19	60	80

\* With very large inoculum.

The lag phase of partially adapted cells cannot be attributed to dilution of growth factors carried over in the inoculum, since the total populations in the training media were not less than normal. Table 3 shows that  $L$  is a very variable quantity, quite apart from the known and controllable influence of the age of the parent culture. The values for parallel inocula from the same parent culture do not show much variation amongst themselves, but strains maintained in broth seem to show slow and fairly steady fluctuations, which, for acetate and succinate at least, are more or less parallel. In spite of these variations in the initial value of  $L$ , it always remains many times larger than the corresponding values for transfer into media towards which the cells are adapted. In the latter it remains short and constant.

This difference is understandable, since the final value depends upon stable, steady states in the cell, whereas the initial value depends upon the accidents of the previous cell history.

The behaviour with the other substrates mentioned in table 3 follows the same general pattern as that described.

Concurrently with the diminution of  $L$  the growth rate of cells usually improves during the course of adaptive training. The growth curves for the first few subcultures in a strange medium are often of rather complex form. Those for the first subculture were measured for acetic acid, succinic acid,  $\alpha$ -keto-glutaric acid and fumaric acid

media. With acetic acid a slow period of growth was followed after a pause by a rather faster one. This behaviour is noted in table 5 by bracketing two values for the mean generation time.

TABLE 5. VARIATION OF MEAN GENERATION TIME DURING ADAPTATION

carbon source	number of previous subcultures	mean generation time (min.)
acetic acid	{ 0	185, 220, (250, 135), (270, 125), (250, 130)
	{ 6	157
	{ 8	136
	{ 19	95, 87, 92
	{ 59	85
succinic acid	{ 0	450, 170, 160, 145, 135, 120, 120, 114, 100
	{ 1	108, 83, 76, 76, 70, 58
	{ 5	92
	{ 10	70
	{ 19	126
	{ 29	55
	{ 34	64, 54
	{ 44	54, 51, 50, 50, 45
fumaric acid	{ 0	95, 130, 105, 80, 77
	{ 9	47, 47, 41, 43
$\alpha$ -keto-glutaric acid	{ 0	200
	{ 1	120, 68

With malonic acid, the tartaric acids, glutaric acid and adipic acid the growth rate was not determined, but was obviously slow. D(-)-Arabinose is exceptional in that it supports growth with a mean generation time of 75 min. from the first subculture, and even after *L* has fallen to the minimum value of zero (Jackson & Hinshelwood 1948).

Adaptation of type (b) is typified by what may be its sole representative amongst these compounds—the training of cells towards glycerol. In the first subcultures the growth curve consists of two parts, an initial slow phase being superseded by a more rapid one. During the course of training the transition occurs earlier and earlier, until finally the culture grows throughout at a uniform rate. *L* is very small at all stages. The fully trained cells have a mean generation time of 33 min. The slow part of the growth curve is normally typified by values between 65 to 90 min. (Cooke & Hinshelwood 1947).

Fresh experiments with *Bact. lactis aerogenes* in glycerol and with very large inocula have confirmed this behaviour. On the first subculture the cells grew with an initial mean generation time of 65 min. and on the fifth subculture with one of 35 min.

Cells transferred from glucose are fully adapted to pyruvic acid, lactic acid DL-malic acid, citric acid, *cis*-aconitic acid, probably to inositol and glyceric acid and nearly so to L(+)-arabinose and D-xylose. For cells of appropriate age *L* is zero at the first subculture, and the rate is optimal from the beginning. The absence of any significant training phenomena is shown in table 6.

TABLE 6. GROWTH RATES UNAFFECTED BY TRAINING

carbon source	initial lag (min.)	lag after training (min.)	initial mean generation time (min.)	mean generation time after training (min.)
lactic acid	0, 0, 150, 120	60, 45, 40	53, 53, 48, 45, 64	44, 41, 56
pyruvic acid	0-60	0-50	47, 48, 43, 40	40, 43, 44
malic acid	150, 60, 0	150, 140, 130	36	36
citric acid	> 180 or 30	> 100 or 30	45 or > 60	45 or > 60
<i>cis</i> -aconitic acid	similar to citric acid			
L-arabinose	0-60	0-60	—	33
D-xylose	0-60	0-60	—	33
glyceric acid	120	—	—	—
inositol	120	—	—	—
glucose	—	0-60	—	33

With substrates towards which the cells require training, the growth rate during the first few subcultures is almost as variable as the lag. The growth rate of fully adapted cells is a reproducible and fundamental property.

TABLE 7. MEAN GENERATION TIMES OF CELLS IN EQUILIBRIUM WITH THEIR MEDIUM

substrate	mean generation time (min.)
glucose	33
L-arabinose	33
D-xylose	33
glycerol	33
malic acid	36
fumaric acid	42
pyruvic acid	45
citric acid	45 or > 60
succinic acid	50
lactic acid	50
aconitic acid	48 or > 60
maleic acid	68
acetic acid	90

The values of the mean generation times for the principal substrates are given in table 7. The anomalous behaviour of citric acid and aconitic acid will be dealt with later.

#### CROSS-TRAINING PHENOMENA

Adaptation to one substance may affect the growth characteristics in others, as manifested in the length of the lag phase and the growth rate.

Of the three compounds, acetic acid, succinic acid and fumaric acid in this order, training to one involves adaptation to later but not to earlier members of the series. Thus cells trained to succinic acid will grow readily with fumaric acid but not with acetic acid (table 8).

TABLE 8. CROSS-TRAINING RELATIONS

training		test		
medium	number of subcultures	with acetic acid lag (min.)	with succinic acid lag (min.)	with fumaric acid lag (min.)
series A:				
glucose only	—	840	840	—
	1	720	120	—
	1	420	180	—
acetic acid	1	330	150	—
	1	240	90	—
	40	0	120	90
series B:				
glucose only		1500	1020	390
		1200	1080	390
		1020	2160	390
	1	720	120	—
	1	1200	270	—
succinic acid	2	—	200	70
	2	—	100	100
	2	—	—	60
	1	1200	2160	40
fumaric acid	1	1020	2100	40
	1	—	2040	—

Confirmation of the fact that acetic acid-trained cells are partially adapted towards succinic acid was provided by measuring the growth rate of cells, trained by twenty-three subcultures with acetic acid, and then inoculated into the succinic acid medium. The mean generation time was found to be 58 min., which is to be compared with values normally found for untrained cells of 100 to 180 min., and with 50 min. for cells trained to succinic acid.

Training to maleic acid appeared to have only a slight effect on growth with fumaric acid. The mean generation time in the latter was 80 to 92 min. compared with values of 130 min. for the untrained cells, and with 42 min. for the fully trained cells.

#### MIXED SUBSTRATES

The growth rate in a glucose medium was found to be unaffected by the addition of citric acid, malic acid, succinic acid, fumaric acid or *cis*-aconitic acid. Acetic acid exercises a definite inhibitory effect which is, however, less if the cells have been previously trained to it.

*L* in the glucose-ammonium sulphate medium is considerably affected by various small additions. It is increased by tartronic acid, propionic acid and acetic acid in high concentration. With tartronic and propionic acids the inhibition is occasionally great enough to stop all growth, but with more dilute propionic acid (1 g./l.) the lag phase is lengthened only from 2 to 3 hr. The lag shown when an ageing glucose culture is subcultured into the standard glucose medium may be appreciably shortened by addition of various acids, as shown in tables 9 and 10.

TABLE 9. REDUCTION OF LAG BY CARBOXYLIC ACIDS

Concentration of added acid = 0.25 g./l.

The age of a culture is reckoned as zero when the count equals 1 million cells/ml. (A fully grown culture has a count of about  $10^8$  cells/ml.)

age of parent (min.)	lag in glucose alone (min.)	lag in glucose + citric acid (min.)	lag in glucose + <i>cis</i> - aconitic acid (min.)	lag in glucose + malic acid (min.)
10	156	54	54	66
45	126	20	32	60
75	152	25	36	42
110	102	18	30	30
167	60	10	0	0
260	0	0	0	0
330 (fully grown)	48	0	0	36
1320	70	40	40	40

age (min.)	glucose alone	+ lactic acid	+ pyruvic acid	+ citric acid	+ malic acid	+ fumaric acid	+ succinic acid	+ acetic acid
- 60	172	128	130	99	85	105	105	60
- 30	154	100	95	68	55	73	74	26
0	103	79	69	50	42	57	74	5
114	60	55	48	44	17	46	40	12
220	38	31	30	18	8	—	—	57
290	24	10	10	5	0	15	10	10
1330	541	432	326	266	278	306	298	293
1390	634	372	383	303	361	342	351	468
1590	934	600	600	500	520	512	505	830
2090	1680	1080	1080	880	960	—	—	900

TABLE 10

addition to glucose media	lag (min.)	lag as % lag in glucose
series A:		
—	370	100
citric acid	—	87
succinic acid	—	75
acetic acid	—	11
tartronic acid	—	103
D-tartaric acid	—	92
formic acid	—	100
series B:		
—	1410	100
citric acid	—	84
succinic acid	—	84
acetic acid	—	92
D-tartaric acid	—	84
tartronic acid	—	100
formic acid	—	100

Combinations of acids were never more effective than the more active one alone (table 11).

TABLE 11

addition to glucose media	lag (min.)	lag as % lag in glucose
—	1280	100
citric acid	870	68
pyruvic acid	970	76
malic acid	977	76
all three	916	71

The approximate order of effectiveness is:

- (1) Malic acid, citric acid, succinic acid and fumaric acid, *cis*-aconitic acid.
- (2) Pyruvic acid, lactic acid.
- (3) Acetic acid, D-tartaric acid.
- (4) Tartronic acid and formic acid (which appear to have no positive effect).

If the concentrations of acetic acid and tartronic acid are increased (to about 3 g./l.) inhibition appears.

The above results may be compared with the effect of added 'heart-broth' on an inoculum from an old parent culture (table 12).

TABLE 12

test medium	lag (min.)
glucose + heart-broth	1680
glucose + citric acid	3780
glucose alone	6000

There are obviously substances in 'heart-broth' far more effective than the acids. (The mean generation time in 'heart-broth' is 18 to 20 min. compared with 33 min. in glucose-ammonium sulphate.)

Experiments were also carried out with differing concentrations of citric and succinic acids (table 13).

TABLE 13. INFLUENCE OF CONCENTRATIONS

concentration added acid moles/l.	lag with citric acid added		lag with succinic acid added	
	min.	% shortening	min.	% shortening
0	143	0	94	0
$1.72 \times 10^{-7}$	130	9.1	88	6
$1.03 \times 10^{-6}$	152	-6	39	59
$6.7 \times 10^{-6}$	145	-2	52	45
$3.7 \times 10^{-5}$	115	19.5	39	59
$2.2 \times 10^{-4}$	87	39	68	25
$1.84 \times 10^{-3}$	74	48	28	70
$1.34 \times 10^{-3}$	72	50	45	52
$5.55 \times 10^{-3}$	79	45	23	75

Citric acid seems to bring about its full effect at concentrations greater than  $4 \times 10^{-4}$  M, and succinic acid above  $10^{-6}$  M.

THE NATURE OF THE LONG INITIAL LAG PHASE

The lag in the succinic acid medium is decreased either by an increase in the size of the inoculum, or by preliminary growth in the actual medium upon traces of added glucose, whereby cell count increases and metabolites are turned out into the solution (table 14).

TABLE 14. VARIATIONS OF LAG IN SUCCINIC ACID

Total volume of medium = 25 ml.								
ml. glucose (50 g./l.)	...	...	0.0	0.005	0.025	0.125	0.005	10
ml. sodium succinate (20 g./l.)	...	...	5	5	5	5	1	0
initial count (10 <sup>6</sup> /ml.)			0.5	0.5	0.5	0.5	0.5	0.5
count after growth on glucose			0.5	14.5	70	350	14.5	1000
lag before glucose is utilized (min.)			120	120	120	120	120	120
lag before succinate is utilized (min.)			5400	4800	3600	2100	4800	—

Experiments made with a washed suspension of cells show that both these effects are important (table 15).

TABLE 15. EFFECT OF INOCULUM SIZE AND OF GROWTH WITH TRACES OF ADDED GLUCOSE UPON LAG ATTENDING FIRST SUBCULTURE IN SUCCINIC ACID

initial count (10 <sup>6</sup> /ml.)	count after growth on added glucose (min.)	lag after growth on glucose (min.)	lag in medium free from glucose (min.)	lag in glucose (min.)
0.5	560	1560 to 2220	—	150
500	—	—	3120 to 3600	—
0.5	280	2280	—	150
250	—	—	3840	—
0.5	110	3000	—	160
100	—	—	4920	—
0.5	56	3000	—	160
50	—	—	5220	—
0.5	11	2700	—	150
10	—	—	5460	150
2.0	—	—	4380	170
0.5	—	—	5580	130

Although a considerable reduction in the lag occurs, a limit is reached, where neither increase in count nor provision of 'glucose' metabolites causes further shortening. A certain intrinsic readjustment of the cells appears to be necessary.

SPECIAL CHARACTERISTICS OF GROWTH IN CITRIC ACID

Two types of growth occur with citric acid, one characterized by a mean generation time of 40 to 48 min., the second by one greater than 59 min. The former usually appears when the lag phase has exceeded 100 min., the latter when the lag has been short.

On the first subculture from a dilute glucose medium into citric acid the curve showing the lag as a function of the age of the parent has the form in figure 3. The greatest value for the lag shown by young cells in citrate is usually about 100 min. (that is, less than for glucose). This is always associated with growth at the slow rate. As the cell count of the parent medium increases the lag in citric acid drops to

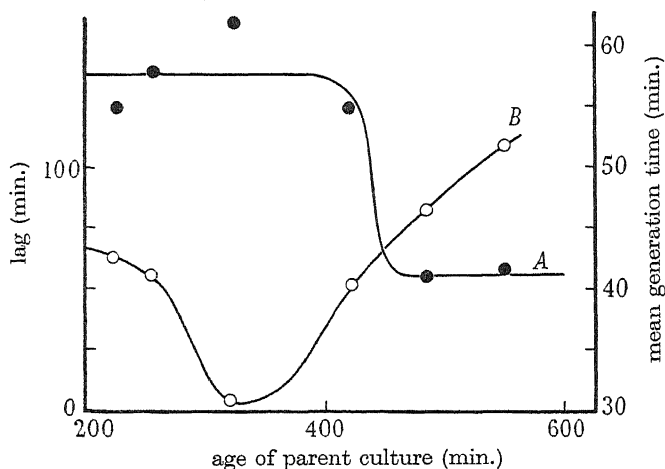


FIGURE 3. Variation of lag and of mean generation time with age of parent culture for growth in citric acid medium. *A*, mean generation time in citric acid medium; *B*, lag in citric acid medium.

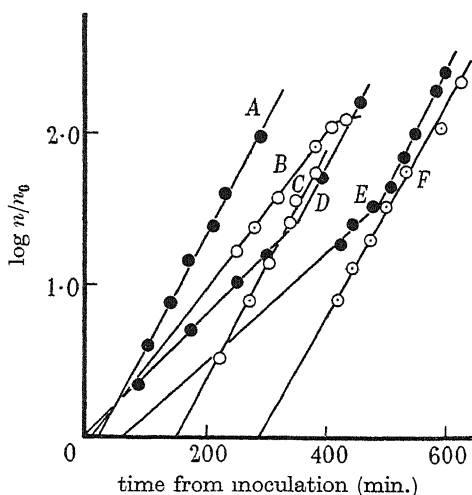


FIGURE 4. Forms of growth curves in citric acid medium. *A*, rapid growth, small lag, transfer from actively growing parent culture, in citric acid; *B*, slow growth, short lag (ex citric acid medium); *C*, rapid growth, long lag (ex citric acid medium); *D*, broken growth curve (ex citric acid medium); *E*, broken growth curve (ex parent culture in glucose); *F*, rapid growth, long lag (ex parent culture in glucose).

a minimum value between 0 and 50 min., and subsequently increases rapidly to about 300 min. Cells subcultured before this increase grow at the lower rate; those subcultured after grow at the higher. Cells subcultured near this point sometimes give broken growth curves, starting to grow almost at once at the lower rate, then suddenly showing a transition to the higher rate (figure 4).



On later subcultures two modes of growth are still found. As before, the low growth rate is always associated with a short lag phase. The higher rate, however, is now sometimes found after a short lag, when the cells have been subcultured from a 'minimum lag' parent growing at the high rate.

Table 16 gives some typical values of lag and mean generation time.

TABLE 16. FIRST SUBCULTURE IN CITRIC ACID

lag (min.)	mean generation time (min.)	lag (min.)	mean generation time (min.)
156	46	0	60
284	47	43	85
289	40	110	60
292	40	117	61
303	37	0	60
365	43	0	62

#### SUBSEQUENT SUBCULTURES

higher rate		lower rate	
lag (min.)	mean generation time (min.)	lag (min.)	mean generation time (min.)
210	40	0	79
180	39	0	75
190	40	0	81
240	38	0	90
197	40	0	60
113	42	0	72
132	42	10	58
129	45	60	67
100	45		
84	45		
50	43		
18	40		

#### INHIBITION BY ACETATE OF GROWTH IN GLUCOSE

Addition of sodium acetate to the normal glucose medium slows down the growth rate. The inhibition is greatest with untrained cells, but even cells trained to acetate still show some inhibition (table 17).

#### LAG PHASE AND ADAPTIVE PHENOMENA

The commonest manifestation of adaptation is the removal by serial subculture of the need for a very long lag phase in the utilization of a given compound.

What seems to occur during the lag is the building up of the steady state where all the intermediates required for growth are present in the necessary proportions. In a closely interlocking system of processes the absence of either of two intermediates

TABLE 17. INHIBITION BY ACETATE

	untrained cells			trained cells (20 subcultures)	
	normality of acetate, M	mean generation time (min.) in glucose	33 + 78 M	normality of acetate, M	mean generation time (min.) in glucose
1	0	33	33	0	33
2	0.063	37.5	37.5	0.063	36
3	0.126	42.5	43	—	—
4	0.252	55	53	0.252	49
5	0.635	83	82.5	1.008	59
6	1.27	123	132	—	—

may hinder the reaction by which the other is built up, small quantities of *A* being needed to prime, as it were, the formation of *B* and vice versa. This could explain the extreme length of the lag in certain examples.

This mutual dependence of processes, characteristic of cyclic systems, is evidenced by the fact that the carboxylic acids are capable of shortening the lag in glucose media, even when they themselves, as with succinic acid, are by no means capable of starting off rapid growth alone. On the other hand, the fact that mixtures of various acids are no more effective than the best single one shows that in proper circumstances they are interconvertible or mutually replaceable.

The provision of intermediates in small amount for the purpose of priming cyclic processes, although important, is by no means the whole story. Sometimes the poor utilization of a new substrate is connected with the feeble functioning of an enzyme system which requires development by actual building of fresh cell material. This is illustrated, with a new substrate, by the failure of glucose additions to remove more than a fraction of the lag which normally occurs on transfer, for example, to succinic acid, and, in another way, by the corresponding inability of 'heart broth' to remove completely the lag which occurs when an inoculum from an ageing parent is transferred even to a fresh supply of its normal glucose medium. In the first case, certain enzyme systems must be developed beyond their normal capacity, in the second decayed enzyme systems must be rebuilt.

#### RECAPITULATION OF RESULTS

The essential results of the foregoing experimental study may be recapitulated as follows:

(a) To some of the many carbon compounds which cells of *Bact. lactis aerogenes* may utilize they are already fully adapted on transfer from a glucose medium. To others they require adaptation, usually for the removal of a very long initial lag, or, less frequently, for the reduction of a long mean generation time.

(b) Adaptation to an earlier member of the series acetic acid, succinic acid, fumaric acid entails that to a later member but not vice versa. This gives a direct indication of the order in which these substances are used.

(c) Growth curves in media with citric acid or aconitic acid have complex forms suggesting alternative sequences of growth reactions.

(d) Appreciable, though by no means unlimited reductions in the lag in glucose media are brought about by additions of various carboxylic acids, including those to which the cells are not adapted.

Several of the questions raised in the introduction to this paper have received a direct answer in the above recapitulation. The general problem of the significance of the tricarboxylic acid cycle for the growth of *Bact. lactis aerogenes* will be considered in part II. It is evident at once that growth-supporting facility bears no relation to the number of carbon atoms or to the functional groups in the molecule. (On the other hand most, if not all, of the compounds which support growth well will either be found to occur in the tricarboxylic acid cycle and allied systems or might be supposed capable of conversion to those which do. Some of those in the cycle are, however, only utilizable after adaptation of the cells.)

For the more detailed consideration of these matters the steps and mechanism of the tricarboxylic acid cycle itself, and the relation of certain other compounds to it must be examined. This general theoretical discussion constitutes the subject of part II.

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# The utilization of carbon sources by *Bact. lactis aerogenes*

## II. The significance of the tricarboxylic acid cycle

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The kinetics of a model related to the 'tricarboxylic acid cycle' are examined, and the significance of this system and of related mechanisms for the growth of *Bact. lactis aerogenes* is considered in the light of the experimental evidence from part I.

### INTRODUCTION

In the present paper certain kinetic aspects of the results recorded in part I will be considered.

It is well established that pyruvic acid is one of the key breakdown products of carbohydrate in yeast and tissue metabolism. It is more highly oxidized than glucose, and in fermentation reactions is finally reduced to such substances as lactic acid or alcohol. In aerobic metabolism, however, pyruvic acid may be completely oxidized to carbon dioxide for which a mechanism has been proposed in the tricarboxylic acid cycle (Krebs 1943) shown in figure 1.

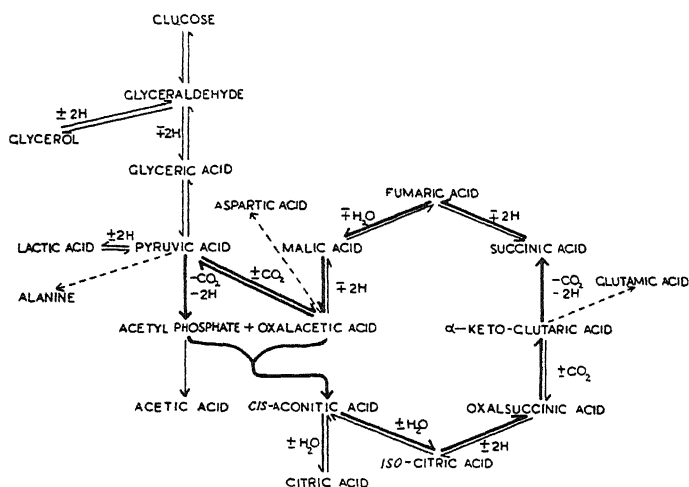


FIGURE 1. Tricarboxylic acid cycle.

Pyruvic acid undergoes two reactions:

(a) By  $\beta$ -carboxylation to form oxalacetic acid which is also regenerated at the end of the cycle, and thus serves as a catalyst.

(b) To provide by oxidative decarboxylation some active 2-carbon compound, probably acetyl phosphate, which condenses with the oxalacetic acid to form *cis*-aconitic acid. By a series of hydrations, dehydrogenations and decarboxylations this

last substance is converted in turn to the following series of compounds: *iso*-citric acid, oxalsuccinic acid,  $\alpha$ -keto-glutaric acid, succinic acid, fumaric acid, malic acid and thence back to oxalacetic acid. Starting with one molecule of pyruvic acid and one of oxalacetic acid the net reaction is the oxidation of the former to water and carbon dioxide and the regeneration of the latter. The cycle from succinic acid onwards corresponds to the catalytic system of Szent-Györgyi (1935, 1936). Besides the main reactions of the cycle there are important side branches to it, such as the formation of alanine, aspartic acid and glutamic acid from their keto-acid precursors pyruvic acid, oxalacetic acid and  $\alpha$ -keto-glutaric acid. These are constituents of protein, the building up of which may well depend partly upon the formation of these compounds. Another important branch of the system is provided by the aconitase system which keeps citric acid in equilibrium with the *cis*-aconitic acid and *iso*-citric acid formed in the cycle.

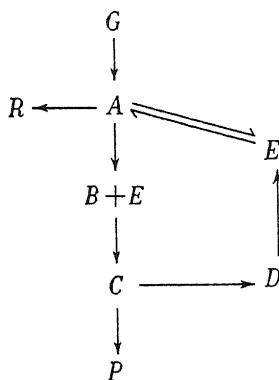


FIGURE 2. Simplified model of cycle.

All the steps in the cycle may be supposed to be reversible under aerobic conditions except the oxidative decarboxylations, but the only hydrogenations which appear to proceed with any rapidity under these conditions are those connecting the Szent-Györgyi system from oxalacetic acid to succinic acid. In effect then the system during aerobic carbohydrate metabolism is supposed to work only in the direction of the heavy arrows in figure 1. (Some of the compounds indicated in this scheme actually occur in the form of phosphorylated derivatives, but this does not affect the general relationships.)

From the kinetic point of view it is interesting to see how the formation of the amino-acids necessary for protein synthesis depends upon the rates at which the various stages of the cycle can proceed. Accordingly a simplified model (figure 2) has been set up which possesses all the essential features of the tricarboxylic acid cycle. With some simple assumptions, the rate of formation of two typical amino-acids (corresponding in fact to alanine and glutamic acid) has been expressed in terms of the different rate constants when various members of the cycle are used as the sole carbon source.

## MODEL OF THE CYCLE

*A* plays a part equivalent to that of pyruvic acid, and the process by which it is formed from *G* may be taken as representative of the formation of the pyruvic acid from glucose. *B* corresponds to the (probably phosphorylated) acetic acid reacting in the cycle with a derivative of oxalacetic acid to give 6-carbon compounds which are degraded and oxidized by way of succinic and fumaric acids to malic acid and oxalacetic acid. All the essentials of the reactions in figure 1 are included in the above scheme, except that certain stages have been telescoped. *E* can be looked upon as the representative of malic acid or oxalacetic acid, *D* of succinic or fumaric acid. Formation of amino-acids which are drained off for the purposes of synthesis and growth is represented by the processes  $A \rightarrow R$  and  $C \rightarrow P$  respectively, which are analogous to the formation of alanine from aspartic acid, and glutamic acid from  $\alpha$ -keto-glutaric acid respectively. All the main types of reaction are thus included in the hypothetical cycle, and the general structure of the system corresponds closely to that of the somewhat extended series of figure 1. The direct interconversion of *E* and *A* must be postulated: otherwise the cycle could not function continuously during growth, since the removal of *C* to give *P* would cause a steady diminution of *E* and thus of the rate of operation of the step  $B + E \rightarrow C$ .

In developing equations for the kinetics of these processes we shall adopt the following assumptions and conventions.

$k_{AB}$  denotes the rate at which *A* is converted into *B*, *a* may be the actual concentration of the substance *A*, or, for a reaction occurring at the surface of an enzyme it may approach a limiting value independent of the concentration. *W* indicates that the substance goes to waste.

In general, a given intermediate has several different possible fates, *C*, for example, may be converted into *D*, or into *P* or it may be lost by diffusion, or in reactions irrelevant to the cycle of processes under consideration. We shall assume that, in general, all these changes are proportional to the same function of the concentration, so that  $\Sigma k_X = k'_X$ , as, for example,

$$k_{CD}c + k_{CP}c + k_{CW}c = k'_C c.$$

The fraction proceeding by a particular route will be designated *x*, so that, for example,  $k_{CD}/k'_C = x_{CD}$ .

The reaction  $B + E \rightarrow C$  calls for special consideration. In general, its rate may be assumed to depend chiefly upon the concentration of that intermediate which is in defect, that is, either upon *b* or upon *e*. *b* and *e* in turn depend upon the other constants of the cycle. A general expression assumes a very cumbrous form. It will therefore be convenient to consider two separate cases, one where the velocity is  $k_{(B)E}e$  and one where it is  $k_{B(E)C}b$ .

It will be assumed that in the cell a steady state is established for the concentrations of the various active intermediates, and that these suffer only relatively slow secular changes as the concentrations of the primary substrates change and the medium conditions alter. In view of the minute scale of cell phenomena this seems entirely justified.

G AS CARBON SOURCE

The equations of the steady state assume the forms

$$-dG/dt = k_0, \quad (1)$$

$$dA/dt = k_0 + k_{EA}e - k'_Aa = 0, \quad (2)$$

$$dB/dt = k_{AB}a - k_{(B)EC}e - k''_Bb = 0, \quad (3a) \text{ (case } a)$$

where  $k''_Bb$  measures the loss of  $B$  other than by reaction with  $E$ ,

or 
$$dB/dt = k_{AB}a - k'_Bb = 0, \quad (3b) \text{ (case } b)$$

$$dC/dt = k_{(B)EC}e - k'_Cc = 0, \quad (4a)$$

or 
$$dC/dt = k_{B(E)C}b - k'_Cc = 0, \quad (4b)$$

$$dD/dt = k_{CD}c - k'_Dd = 0, \quad (5)$$

$$dE/dt = k_{AE}a + k_{DE}d - k'_Ee = 0, \quad (6a)$$

or 
$$dE/dt = k_{AE}a + k_{DE}d - k_{B(E)C}b - k''_Ee, \quad (6b)$$

$k''_Ee$  measuring the loss of  $E$  other than by reaction with  $B$ .

For the purpose of considering growth phenomena we are interested in the rates of formation of  $P$  and  $R$ , namely,  $k_{CP}c$  and  $k_{AR}a$ .

From the above equations the following expressions are derived by elimination.

For case  $a$ :

$$k_{CP}c = \frac{x_{CP}x_{(B)EC}x_{AE}k_0}{1 - x_{(B)EC}x_{CD}x_{DE} - x_{EA}x_{AE}}, \quad (Ia)$$

$$k_{AR}a = \frac{x_{AR}k_0}{\left(1 - \frac{x_{EA}x_{AE}}{1 - x_{(B)EC}x_{CD}x_{DE}}\right)} = \frac{x_{AR}(1 - x_{(B)EC}x_{CD}x_{DE})k_0}{(1 - x_{(B)EC}x_{CD}x_{DE} - x_{EA}x_{AE})}. \quad (IIa)$$

Although the rate of growth will not depend upon a simple product of the concentrations of  $P$  and  $R$ , it is clear that for effective synthesis neither  $k_{CP}c$  nor  $k_{AR}a$  must fall below a certain level. In this sense the product of these two quantities is a determining factor.

According to this model, for case  $(a)$  with  $G$  as substrate, there can be no satisfactory growth if any of the fractions  $x_{CP}$ ,  $x_{(B)EC}$  or  $x_{AE}$  are very small, on the one hand, or  $x_{AR}$  on the other. The expressions  $(Ia)$  and  $(IIa)$  appear to allow  $x_{AB}$  to be vanishingly small. But if this were so,  $B$  would become deficient and the rate of formation of  $C$  would become dependent on  $b$ . This would be a transition to case  $(b)$ .

For case  $(b)$  the stationary state equations yield

$$k_{CP}c = \frac{x_{CP}x_{B(E)C}x_{AB}k_0}{\{1 - x'_{EA}x_{AE} + x'_{EA}x_{B(E)C}x_{AB}(1 - x_{DE}x_{CD})\}}, \quad (Ib)$$

$$k_{AR}a = \frac{x_{AR}k_0}{\{1 - x'_{EA}x_{AE} + x'_{EA}x_{B(E)C}x_{AB}(1 - x_{DE}x_{CD})\}}, \quad (IIb)$$

where

$$x'_{EA} = k_{EA}/k''_E.$$

According to these the growth rate would vanish if  $x_{AB}$ ,  $x_{AR}$ ,  $x_{CP}$  or  $x_{B(E)C}$  became too small. At first sight  $x_{AE}$  could become zero without adverse effect, but reference to the diagram of the cycle shows that the removal of material in the process  $CP$  must be at least balanced by the supply of  $E$  from  $A$ : otherwise  $E$  would fall and case (a) would operate once more. Here (Ia) shows that growth stops when  $x_{AE}$  is too small.

If the above scheme is taken as a rough model for growth in a medium supplied with glucose, then the satisfactory rates observed with this substrate vouch for adequate values of  $x_{CP}$ ,  $x_{AR}$ ,  $x_{AB}$ ,  $x_{B(E)C}$  or  $x_{B(E)C}$  and  $x_{AE}$ . They do not necessitate good values for  $x_{CD}$  or  $x_{DE}$ . This means that amination of  $\alpha$ -keto-glutaric and pyruvic acids, the conversion of pyruvic to phosphorylated acetic acid, the reaction of the latter with oxalacetic acid or its derivative, and the formation of oxalacetic acid from pyruvic acid must all have high rates. There is, on the other hand, no necessity to infer high rates for the parts of the scheme involving the formation and further reaction of succinic and fumaric, and malic acids.

#### $D$ AS CARBON SOURCE

Further information about these processes may be expected from the study of the system where the substrate provided is not  $G$  (glucose), but  $D$  or  $E$ , that is, succinic or fumaric or malic acid. Consider  $D$  as substrate.

For the new set of stationary state equations, we have  $k_0 = 0$ , and in place of  $k_{DE}d$  we have  $k_{D_0}$ , since with the large excess of  $D$  the enzymes will be saturated and the rate of formation of  $E$  from  $D$  will be constant. With these modifications the solutions are as follows:

Case (a)  $BE \rightarrow C$  depending upon  $e$ :

$$k_{CP}c = \frac{x_{CP}x_{B(E)C}k_{D_0}}{1 - x_{EA}x_{AE}}, \quad (\text{III } a)$$

$$k_{AR}a = \frac{x_{AR}x_{EA}k_{D_0}}{1 - x_{EA}x_{AE}}. \quad (\text{IV } a)$$

Case (b)  $BE \rightarrow C$  depending upon  $b$ :

$$k_{CP}c = \frac{x_{CP}x_{AB}x'_{EA}x_{B(E)C}k_{D_0}}{1 - x'_{EA}x_{EA} + x'_{EA}x_{AB}x_{B(E)C}}, \quad (\text{III } b)$$

$$k_{AR}a = \frac{x_{AR}x'_{EA}k_{D_0}}{1 - x'_{EA}x_{AE} + x'_{EA}x_{AB}x_{B(E)C}}. \quad (\text{IV } b)$$

$x'_{EA}$  has the meaning  $k_{EA}/k''_E$ .

Adequate values of certain of the quantities in the numerators of (IIIa), (IIIb), (IVa) and (IVb) are provided for by the postulate that growth is good with  $G$  as substrate. The only conditions, according to the model, for very low rates with  $D$  would be extremely small values of  $k_{D_0}$  on the one hand, or, on the other, of  $x_{EA}$  or  $x'_{EA}$ , which do not appear in the numerators of (Ia), (Ib), (IIa) or (IIb). The former would imply an enzyme system inactive for the consumption of  $D$ , the latter an extremely small rate of conversion of  $E$  to  $A$  or an extremely wasteful loss of  $E$  by diffusion.



CONSIDERATION OF INDIVIDUAL CARBON SOURCES

Malic acid as substrate would yield oxalacetic acid and thus play a role similar to  $E$ . Growth with malic acid is good, nearly though not quite equal to that with glucose. Moreover, no marked training phenomena are in evidence. Unless, therefore, the massive concentrations of malic acid prevailing when it is actually provided as a substrate have an influence on the process  $E \rightarrow A$ , of a quite different order from those which the corresponding concentrations of succinic acid have on the process  $D \rightarrow E$ , when this acid itself plays the role of added substrate  $D$ , slow growth with succinic acid very probably implies a low value of  $k_{D_0}$ , and therefore of  $k_{DE}$ .

Returning now to the processes involved in glucose utilization, we see that  $E$  is formed by two routes, direct from  $A$  and by way of  $D$ . The ratio  $k_{DE}d/k_{AE}a$  is easily found and depends upon  $x_{DE}$ . If the latter is small most of the  $E$  is in fact formed direct from  $A$  rather than by way of the circuit.

Now although the assumption of abnormal losses of  $E$  cannot be entirely discounted, and the low value of  $k_{DE}$  is probable rather than proven, an inversion of the argument leads to very interesting results. If  $k_{DE}$  is in fact small, then with glucose as substrate, most of the  $E$  will in fact be formed from  $A$ . That is, the oxalacetic acid will come directly from pyruvic acid and not by the circuit through succinic acid. This would provide a very good reason why cells normally grown in glucose should be highly untrained towards succinic acid, showing a very long initial lag, as they in fact do.

That cells should be able to grow in glucose with their succinic acid utilizing system relatively undeveloped, while possessing such a system ready for expansion when circumstances demand it, is, on the view outlined, good evidence that the structure of the circuit by which the various carbon compounds are related is of the form given in figure 1, with the double route to oxalacetic acid from pyruvic acid.

On the view discussed we can understand the behaviour of the cells with succinic and fumaric acids. The good growth with malic acid only requires easy conversion of malic to oxalacetic acid.

The behaviour of some of the other compounds can be interpreted by taking into account certain factors connected with, though not actually expressed in, the model system which has been described.

Pyruvic acid is represented by the substance  $A$ , and according to a literal interpretation of the scheme should permit growth at least as rapid as that with glucose. In point of fact, although the cells are fully trained from the start, the mean generation time is never less than about 43 min. whilst that with glucose is 33 min. In principle, there are two possible types of explanation for this behaviour. Either substances lying on the route between glucose and pyruvic acid play a significant part in the growth of the cell or in some way the pyruvic acid itself exerts an inhibition. As to the first possibility, the formation of polysaccharides or of 5-carbon compounds such as pentoses might be required. As to the second, the increased concentration of  $A$  could inhibit the conversion of  $C$  into  $P$  by competing too keenly for some essential nitrogenous intermediate used by both  $C$  and in its own conversion to  $R$ . Excess of pyruvic acid could also act directly by blocking the enzyme positions responsible for converting  $C$  into  $P$ .

According to the metabolic scheme lactic acid would be first converted into pyruvic acid. The cells are fully trained to lactic acid, and the mean generation time is rather greater than with pyruvic acid. If the conversion of the lactic acid were slower than the rate at which the pyruvic acid can be metabolized, this would follow naturally.

The substance *B* in the scheme would represent either acetic acid or acetyl phosphate. Thus *B* alone possesses only two carbon atoms, and before it can pass round the cycle some synthetic reaction must occur. One possibility is a reversal of the reaction by which, normally, oxidative decarboxylation occurs. This could be followed by  $\beta$ -carboxylation of the pyruvic acid to give oxalacetic acid (*E*). In aerobic cultures the reversal of an oxidative decarboxylation does not seem very likely, nor would it explain the cross-training to succinic acid. Alternatively, two molecules of acetic acid could couple to give succinic acid. This mechanism would involve an oxidation which is not unlikely. It would explain the cross-training relationship (see part I, table 8) and also the long initial lag in acetic acid and the mean generation time of 90 min. which is greater even than that in succinic acid.

The behaviour of malonic acid appears to be rather similar to that of acetic acid, which it very readily yields by decarboxylation.

The substance *C* may be supposed to represent the series of compounds from *cis*-aconitic acid to  $\alpha$ -keto-glutaric acid.

The actual behaviour of the cells towards *cis*-aconitic acid and towards citric acid appears to be closely similar. The dual types of growth with citric acid suggests that there may be two or more ways in which it is utilized. One of these could be by way of succinic acid, though the latter is a poor supporter of growth. The utilization of citric acid would, however, be better than that of succinic acid, since *P*, in the shape of glutamic acid, is tapped off before the slow stage is reached. The glutamic acid, moreover, might have the effect of removing some of the long lag phase associated with succinic acid, and it might even undergo breakdown itself to give substances such as pyruvic acid.

Growth by this route might be expected to resemble growth in  $\alpha$ -keto-glutaric acid, where the mean generation time corresponds fairly closely to that of 'slow growth' in citric or *cis*-aconitic acids.

A second possible route for citric acid would be by the hydrolysis of *cis*-aconitic acid to give both *E*, oxalacetic acid, and thence pyruvic or malic acid and *B*, acetic acid.\* The fast growth rate in citric acid is indeed similar to that in pyruvic acid, the mean generation time being about 45 min. for both. The reversal of the normal metabolic route might well be associated with a certain amount of lag which would explain why the faster mode is associated with the longer lags.

\* Analysis of the anaerobic fermentation products of certain strains of bacteria, including *Bact. lactis aerogenes*, acting on citric acid, indicate that it is first split into acetic acid and oxalacetic acid, the latter being further converted into a variety of products (Slade & Werkman 1941; Brewer & Werkman 1939; Deffner & Franke 1939). Under anaerobic conditions this route would appear to be the most likely, but this work referred to does not seem to rule out the possibility that under aerobic conditions the metabolic route might be rather different, for example, via  $\alpha$ -keto-glutaric acid as in the tricarboxylic acid cycle.

As has been remarked, the similarity of growth with  $\alpha$ -keto-glutaric acid to the slow growth with citric acid is consistent with the position that the former occupies.

Of the other substances investigated only glyceric acid and glycerol bear any close relationship to this metabolic scheme. Glyceric acid lies on the route between glucose and pyruvic acid, and the short lag of the cells in this medium is in keeping with this position. Glycerol is a stage removed from the direct reaction route, a fact which may explain why the growth rate is initially low but reaches the glucose value after adaptation.

Maleic acid should be somewhat similar to fumaric acid. Actually growth in maleic acid is rather worse, and training of the cells to maleic acid only causes a very slight improvement in the initial growth rate in fumaric acid.

Tartaric acid might be supposed to resemble other four-carbon acids. Growth in D-tartaric and *meso*-tartaric acids appears to be rather bad as though there were some awkward steps to be overcome. L-Tartaric acid refuses to support growth, probably for reasons of configuration.

The other substances investigated have no close relationship to the cycle. If they are not sugars or closely related substances they either fail to support growth or only do so very poorly. If they are sugars growth is sometimes good, sometimes poor, according to the stereo-chemistry of the compounds. If the latter is favourable, junction with the glucose route at a point well before pyruvic acid should present no special difficulty.

#### CONCLUSION

In the light of the foregoing the significance of the tricarboxylic acid cycle in the growth processes of *Bact. lactis aerogenes* may perhaps be assessed in the following way.

The different adaptive phenomena, the various growth rates, and the existence of alternative growth mechanisms are most easily interpreted by the assumption of a considerable network of metabolic routes, sections of which may be utilized in various combinations. On this network the connexions indicated by the scheme of the tricarboxylic acid cycle seem in general to represent important lines of traffic. There are, however, numerous by-passes and alternatives. Some sections of the cycle may in certain circumstances be used rather in the reverse than the forward direction, and some stretches appear to be utilized only to a small extent by cells grown in glucose. Nevertheless, the structure of the cycle itself is such as to allow partial rather than full utilization of these stretches. The general conclusion is that the cycle does indicate one highly significant pattern among the carbon compounds which support the growth of the bacterium under study.

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# The utilization of potassium by *Bact. lactis aerogenes*

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As the potassium-ion concentration of synthetic media is decreased the total population ( $n_s$ ) of *Bact. lactis aerogenes* which they support tends towards zero. Added potassium gives nearly linear increases in  $n_s$ . The use of the radioactive isotope shows that the cells take up nearly all the potassium from the medium over wide ranges of concentration.

Sodium cannot replace potassium, and the use of the active isotope shows that it is probably not taken up by the cells.

Rubidium replaces potassium with about quarter efficiency, but lithium and caesium have only very small effects.

The potassium appears to play the part of an enzyme-activator, and in glucose-ammonium sulphate media is displaced by the acid formed during growth.

A quantitative treatment of the competition between  $K^+$  and  $H^+$  for an array of negative sites on an enzyme surface is attempted. On the assumption that a certain critical area of  $K^+$ -activated sites is necessary for growth to continue, a relationship between  $n_s$  and the  $[K^+]$  is developed and shown to be in general agreement with the experimental facts.

The relation between  $n_s$  and potassium concentration varies from one medium to another. In glycerol-ammonium sulphate media, where  $n_s$  is insensitive to the initial pH, the amounts of potassium present in the ammonium or sodium salts of the buffer support considerably greater populations than they can in the glucose medium. Further increases, however, demand much greater potassium concentrations, the results being in accord with the view that other products formed in the glycerol medium can displace potassium from the relevant enzyme and limit growth.

## INTRODUCTION

The work to be described is part of a systematic study of the growth and metabolism of the coliform organism *Bact. lactis aerogenes*, and deals with the function of alkali metal ions.

Previous workers have studied the influence of alkali metals on the metabolism of yeasts (Pulver & Verzar 1940; Rothstein & Haege 1943; Conway & O'Malley 1943, 1944), and of *Bact. coli* (Leibowitz & Kupermintz 1942). The results have been summarized by Brooks (1947). The general impression gained from these results is that potassium plays a specific role in the metabolism of sugars by the cells, and that in certain ways, not very clearly defined, there may be antagonism between potassium ions and hydriions.

The general methods of investigation used in the present work involved (a) serial subculture of actively growing cells in media free (except for traces present as impurity, in other reagents) of potassium and sodium ions, lag phase, growth rate and total bacterial population being determined, (b) transfers from cultures starved of alkali ions to media containing known graded additions of the various alkali metal ions, (c) determination of the uptake of radioactive sodium and potassium.

GENERAL RELATIONS BETWEEN GROWTH CHARACTERISTICS AND  
ALKALI METAL-ION CONCENTRATION

A standard culture was prepared in a medium consisting of glucose, ammonium sulphate, magnesium sulphate and a phosphate buffer approximately M/40 in sodium and potassium ions. The cells were fully adapted to this medium. The pH was 7.10. All experiments were made at 40.0° C.

Transfers were made to a similar medium in which the buffer was prepared either from ammonium phosphate, from sodium phosphate or from potassium phosphate, the salts being of the highest degree of purity obtainable.

No difference in the lag phase could be detected as a consequence of any of these changes of medium.

The removal to the utmost extent possible of all sodium from the medium had no influence on the growth rate, but when the potassium-ion concentration fell below a certain value the rate became detectably lower, and in the range 1 to  $2 \times 10^{-5}$  g.ion/l. was approximately linearly dependent upon the concentration (table 1). In a still lower range the growth rate would fall still more but cannot be measured since the total bacterial population then becomes too small.

TABLE 1. DEPENDENCE OF MEAN GENERATION TIME ON ADDED POTASSIUM

K <sup>+</sup> added (g.ion/l. $\times 10^7$ )	mean generation time (min.)
0	60
29	49
58	42
92	36
117	36
146	36
excess	33

It is in fact upon the total population ( $n_s$ ) that the most important effects are observed. Changes in sodium-ion concentration from  $10^{-2}$  to  $10^{-6}$  g.ion/l. produce no detectable regular change in  $n_s$  in presence of M/40-potassium phosphate (table 2).

TABLE 2. TOTAL BACTERIAL POPULATION ( $n_s$ ) AND SODIUM-ION  
CONCENTRATION

Each culture tube contained 10 ml. glucose (50 g./l.); 5 ml. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (5 g./l.); 1 ml. MgSO<sub>4</sub> (1 g./l.); 10 ml. phosphate (M/15 in PO<sub>4</sub><sup>'''</sup>, added as KH<sub>2</sub>PO<sub>4</sub> with KOH to bring pH to 7.10). Sodium added as NaCl.

Na <sup>+</sup> added (g. ion/l. $\times 10^7$ )	$n_s$ (millions/ml.)
0	397
197	395
394	360
788	445
1182	441
1576	475
1970	335
3940	295
5910	433
7880	366
9850	480

After several subcultures in the ammonium phosphate-sodium phosphate buffer, necessary to eliminate the appreciable quantities of potassium transferred with the cell inoculum from the original medium,  $n_s$  fell to a low value, at which it became stabilized. Whether this represented growth independent of potassium, or growth on the traces of potassium present as impurity in the other salts will be considered later.

The effects of changes in potassium-ion concentration are shown in table 3 and figure 1.

TABLE 3. TOTAL BACTERIAL POPULATION ( $n_s$ ) AND POTASSIUM-ION CONCENTRATION

Media as in table 2 except that buffers contained ammonium phosphate (M/15) brought to pH 7.10 with HCl. Varying amounts of potassium and sodium chlorides added.

K <sup>+</sup> added (g.ion/l. $\times 10^7$ )	Na <sup>+</sup> added (g.ion/l. $\times 10^4$ )	$n_s$ (millions/ml.)	K <sup>+</sup> added (g.ion/l. $\times 10^7$ )	Na <sup>+</sup> added (g.ion/l. $\times 10^4$ )	$n_s$ (millions/ml.)
0	0	57	412	1.3	235
206	0	145	412	5.2	235
412	0	225	412	9.9	237
618	0	287	412	19.7	240
824	0	350	412	39.4	235
1030	0	412	412	500	240
0	2	60	824	1.3	366
0	4	62	824	5.2	372
0	6	62	824	9.9	350
0	9.9	57	824	19.7	347
0	13.8	63	824	39.4	350
0	500	63	824	500	345

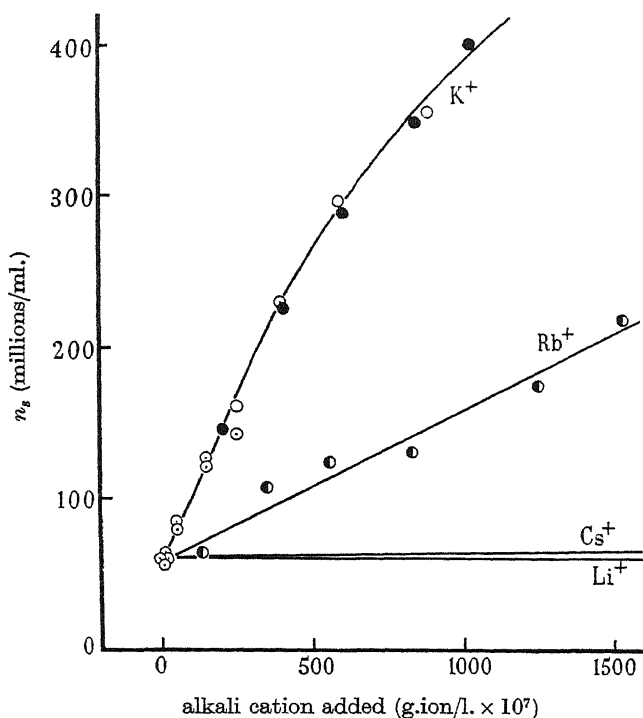


FIGURE 1. Variation of total population with concentration of added alkali cations.

The maximum population is approximately linearly dependent upon the potassium-ion concentration in the range  $10^{-6}$  to  $10^{-4}$  g.ion/l. This is shown in figure 1, which leaves no doubt that potassium is essential for the growth of these cells. The addition of varying quantities of sodium ion produces no change in the population supported by a given potassium-ion concentration (table 3).

Since the media are never less than approximately M/30 with respect to ammonium ion, and, where potassium and sodium are both absent, contain three times this concentration, the possibility of replacement of sodium or potassium by ammonium ion cannot be overlooked. The lowest value of  $n_s$  observed on subculture into sodium or ammonium buffers is 60 million cells/ml. This cannot, however, represent the cell count supported by ammonium ion, since, if the suspension is freed from cells by centrifuging and the solution is reinoculated, growth only proceeds to a count of 20 million, a figure which can be raised as required by addition of potassium ions. The count of 60 million probably represents the growth supported by the potassium present as impurity in the sodium or ammonium phosphates. If figure 1 is linearly extrapolated to  $n_s = 0$  a value of  $110 \times 10^{-7}$  g.ion/l. is found for the concentration of this potassium. Further additions to the media which have been freed of potassium by cell suspensions in the way described give more or less linear increases in count, so that the linear extrapolation to zero values of  $n_s$  is probably justified. When, therefore, the total potassium concentration is required for calculations, this zero value will be included.

Although, as has been shown, sodium will not replace potassium, rubidium will support growth, though with less efficiency than potassium (table 4). Lithium and caesium have very small effects. A comparison of the various alkali ions is shown in figure 1.

Gravimetric analysis of the rubidium chloride showed that it was of a high degree of purity and could not possibly have contained more than a very small fraction of the potassium which would have been necessary to account for the results in table 4.

Experiments made with the radioactive isotope  $K^{42}$ , and which are discussed in the appendix, showed that the cells take up potassium almost completely from the

TABLE 4.  $n_s$  AND ALKALI METAL-ION CONCENTRATION

Media as in table 3.

Rb <sup>+</sup> added (g.ion/l. $\times 10^7$ )	$n_s$
139	64
349	107
556	124
834	130
1251	174
1529	218
ion at $5 \times 10^{-5}$ g.ion/l.	$n_s$
Li <sup>+</sup>	0.2
K <sup>+</sup>	222
Rb <sup>+</sup>	44
Cs <sup>+</sup>	2

medium, and that when growth ceases all but 3 to 4 %, over the whole range of concentrations used, is incorporated into the cells.

Although, as shown by the use of  $K^{42}$ , the selective absorption of potassium by the cells is very pronounced, washing with isotonic (9 g./l.) potassium chloride solution leads to rapid exchange of the active for the inactive isotope. Two conclusions can be drawn from this observation, first that potassium ions can pass freely through the cell membrane, and secondly that intracellular potassium is not very firmly or permanently incorporated in the cell material. \*

Similar washing with sodium chloride removes very little of the active isotope from the cells.

The intracellular potassium not only undergoes exchange with other potassium ions but also suffers displacement by hydrions, at least 80 % being lost from the cells when they are transferred from a buffer at pH 7.1 to one at pH 4.8 (appendix).

The general conclusion of previous workers that the functioning of cellular potassium is antagonized by hydrions is thus confirmed.

This antagonism is more explicitly demonstrated when the total population supported by a given potassium concentration is measured for different initial pH values. Table 5 includes observations at six different potassium concentrations. At each of these a steady decline in  $n_s$  is observed as the hydrion concentration is increased (figure 2).

The question arises whether under conditions where other substrates limit growth, a similar variation with initial pH would be found.

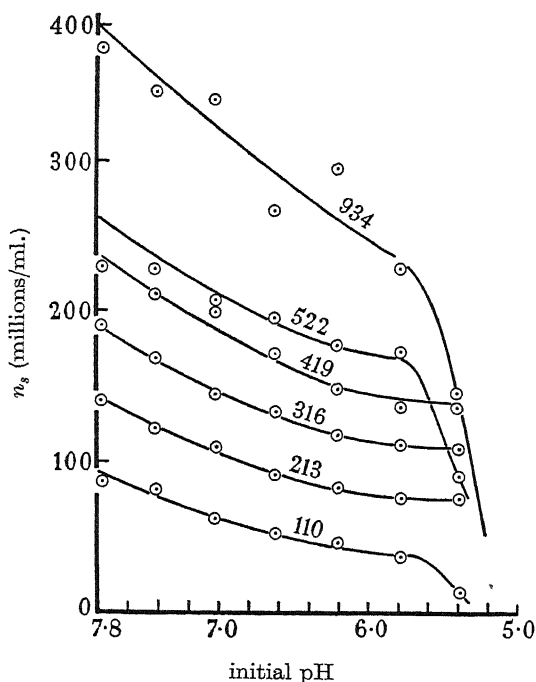


FIGURE 2. Variation of total population with initial pH for various concentrations of added potassium. The numbers against the curves are the values of  $a$ . ( $a$  = concentration in g.ion/l.  $\times 10^7$ .)



TABLE 5. TOTAL POPULATION AND POTASSIUM CONCENTRATION.  
VARIATION OF INITIAL pH

K <sup>+</sup> added (g.ion/l. × 10 <sup>7</sup> )	pH...	Total population						
		7.77	7.41	7.02	6.62	6.20	5.78	5.38
0		86	80	62	51	45	36	13
103		140	121	109	91	82	75	75
206		190	168	144	133	117	111	108
309		230	211	199	172	149	136	145
412		—	227	207	195	177	171	90
824		375	346	340	267	295	228	136

Table 6 shows the total population derived from two low glucose concentrations at different initial pH values, and table 7 shows corresponding results observed in conditions such that exhaustion of ammonia is the limiting factor (figures 3 and 4).

The simplest interpretation of these results is that in the region where  $n_s$  is independent of pH the extent of growth is limited by exhaustion of glucose or ammonia, and in the other ranges it is limited by accumulation of hydrions while glucose and ammonia still remain. The contrast in this respect between the utilization of potassium and that of glucose or ammonia suggests that the intracellular function of the potassium is intimately linked with some process involving the hydrions.

TABLE 6.  $n_s$  and pH (GLUCOSE DEFICIENT)

pH	240 mg./l. glucose	480 mg./l. glucose
	$n_s$ (millions/ml.)	$n_s$ (millions/ml.)
7.84	97	192
7.70	102	197
7.20	103, 108	194
6.78	118, 111	211
6.44	110, 111	188
6.14	109	202
5.86	104	193
5.40	99	183
5.36	95	162
4.96	95	161
4.60	112	161
3.64	5	26

TABLE 7.  $n_s$  AND pH (AMMONIA DEFICIENT)

pH	15.7 mg./l. ammonia	30.1 mg./l. ammonia
	$n_s$ (millions/ml.)	$n_s$ (millions/ml.)
7.70	112	200
7.20	113, 95	194
6.78	118, 104	195
6.44	114, 116	195
6.14	113	190
5.86	114, 122	15
5.36	96, 120	10
4.60	9, 11	9

In the next section an attempt is made to treat theoretically the displacement of potassium by the hydrions formed during growth. In this way an expression will be derived from the relation between  $n_s$  and the potassium concentration, on the basis of certain reasonable assumptions about the role of the potassium in the cell.

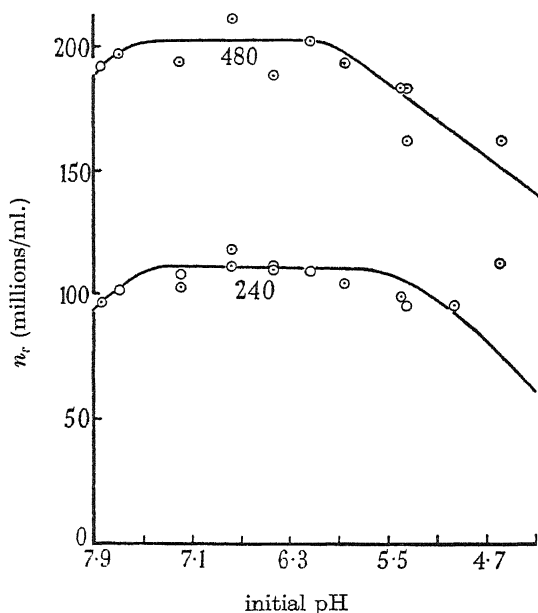


FIGURE 3. Variation of total population with initial pH at two glucose concentrations (glucose exhaustion limiting growth over most of the range). Figures on curves show concentration in mg./l.

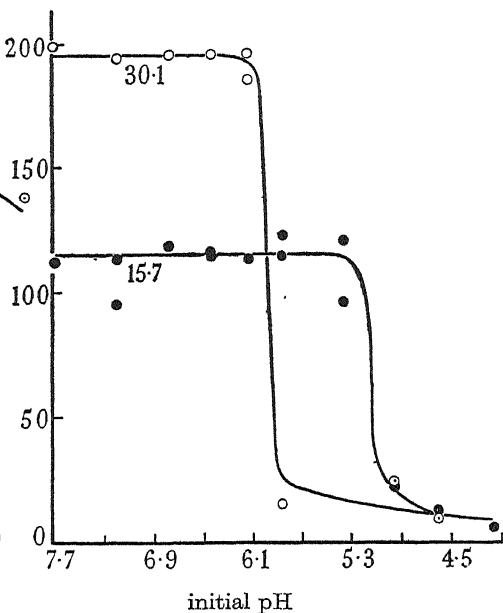


FIGURE 4. Variation of total population with initial pH at two ammonium sulphate concentrations (ammonia exhaustion limiting growth over most of the range). Figures on curves show concentration in mg./l.

### THEORETICAL CONSIDERATIONS

#### (a) *Rate of acid production by the cells*

Growth in glucose results in the production of considerable quantities of hydrion. Various organic acids, such as acetic acid and lactic acid, are formed, and ammonia is removed during nitrogen metabolism, so that complex changes in pH occur.

Before the main problem can be satisfactorily dealt with it is necessary to develop an equation which expresses the hydrion concentration of the medium at any given stage of the growth. The equation to be derived depends upon various assumptions and approximations, but before it is applied, will be tested empirically.

The main buffering action of the medium is taken to be provided by the phosphate ion. At any stage of growth the amount of hydrion produced is represented as equivalent to  $x$  moles of a strong acid, and the change in hydrion concentration is calculated from the law of mass action.

Let  $p$  be the total phosphate in all forms.

Let  $[H^+]_1$  and  $[H^+]_2$  respectively be the initial hydrion concentration and that after the formation of  $x$  moles of strong acid.

Then if  $\chi$  is the appropriate dissociation constant of phosphoric acid

$$\frac{[\text{H}^+]_1 [\text{HPO}_4'']_1}{[\text{H}_2\text{PO}_4']_1} = \frac{[\text{H}^+]_2 [\text{HPO}_4'']_2}{[\text{H}_2\text{PO}_4']_2} = \chi.$$

Since practically all the added hydron appears as  $\text{H}^+$  or  $\text{H}_2\text{PO}_4'$

$$[\text{H}^+]_1 + [\text{H}_2\text{PO}_4'] + x = [\text{H}^+]_2 + [\text{H}_2\text{PO}_4']_2.$$

Also

$$p = [\text{HPO}_4'']_1 + [\text{H}_2\text{PO}_4']_1 = [\text{HPO}_4'']_2 + [\text{H}_2\text{PO}_4']_2.$$

From these relations

$$[\text{H}^+]_1 + \frac{p}{1 + \chi/[\text{H}^+]_1} + x = [\text{H}^+]_2 + \frac{p}{1 + \chi/[\text{H}^+]_2}. \quad (1)$$

Let it now be assumed that each cell produces acid at a constant rate so that

$$\frac{dx}{dt} = k_a n,$$

where  $n$  is the cell count at time  $t$ . When the cell count is rising exponentially

$$n = n_0 e^{kt},$$

where  $n_0$  is the initial count.

$$x = \int_0^t k_a n_0 e^{kt} dt = (k_a/k) (n - n_0).$$

Since in these experiments  $n$  is of the order of a hundred times greater than  $n_0$  we may write

$$x = (k_a/k) n. \quad (2)$$

Substitution in (1) gives

$$[\text{H}^+]_1 + \frac{p}{1 + \chi/[\text{H}^+]_1} + \frac{k_a}{k} n = [\text{H}^+]_2 + \frac{p}{1 + \chi/[\text{H}^+]_2}. \quad (3)$$

Whether or not (1) and (2) individually are exact, (3) can be tested empirically by direct measurement of  $[\text{H}^+]_2$  as a function of  $n$  during growth. There should, for a given initial value  $[\text{H}^+]_1$ , be a linear relation between  $n$  and  $[\text{H}^+]_2 + 1/(1 + \chi/[\text{H}^+]_2)$ .

The results of such a test are shown in figure 5.

The units of the variables in this equation refer to that volume of medium which contains  $p$  g.ions of phosphate. Thus if  $p$ ,  $[\text{H}]_1$ ,  $[\text{H}]_2$  and  $\chi$  are expressed in g.ions/l., then  $n$  must also be converted to millions of cells/l. Measurements were made at two different potassium-ion concentrations to ascertain whether the acid development can be treated independently of the role assumed for the potassium in the cell.

The figure shows, in the first place, that the initial rate of acid production is the same at the two different concentrations of potassium. In the second place, it shows that the plot is roughly linear except when  $n$  is approaching its final stationary value, as shown by the dotted lines in the diagram. Since the marked deviation from linearity occurs at quite different places in the two cultures, it is clearly not due to breakdown of the equations which describe the buffering action, but to failure of the assumption that  $k_a/k$  is constant. When growth is ceasing,  $n$  no longer increases appreciably, but fermentation goes on and produces acid. Even if this acid displaces

potassium from the cells, it cannot of course reduce the count. While roughly 80 % of the cells are being formed the linear relation is approximately followed. We shall therefore regard equation (3) as a reasonable approximation for the purpose in view.

The initial slope of the line in figure 5 gives a value for  $(1/p)(k_a/k)$  of approximately  $1.5 \times 10^{-3}$ ,  $n$  being measured in millions/ml. Since we shall require the volume

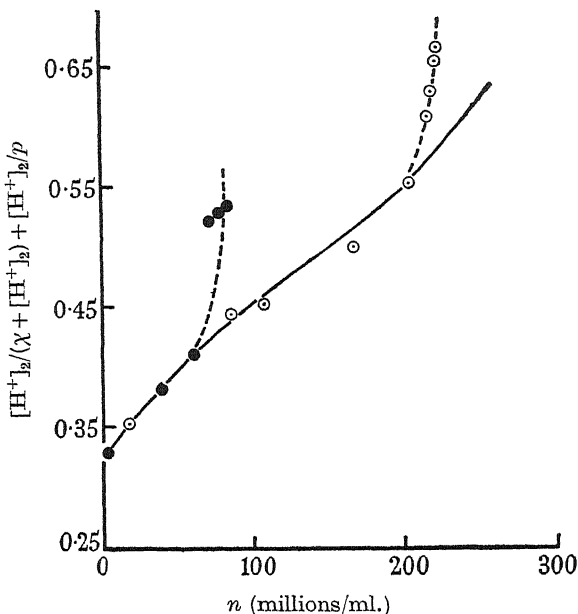


FIGURE 5. Rate of acid production. Solid circles:  $K^+$  added =  $103 \times 10^{-7}$  g.ion/l.,  $n_s = 95$ ; open circles:  $K^+$  added =  $515 \times 10^{-7}$  g.ion/l.,  $n_s = 240$ .

units of  $n$  and  $p$  to be the same, we shall now convert the counts to millions/l., the value of  $(1/p)(k_a/k)$  becoming  $1.5 \times 10^{-6}$ . The slope tends to decrease somewhat as  $n$  increases, showing finally the steep increase already referred to.

In so far as (3) is empirically justified as an approximation, the considerations that follow are independent of the assumptions upon which it was based.

#### (b) Role of potassium ions

The simplest way in which the potassium can be thought of as participating in the chemical reactions of the cell is that its ions should become attached to an array of negative centres in some enzyme for whose functioning they are essential. This hypothesis suggests the mode of antagonism of hydriens, which would be assumed to displace the potassium from the negative centres and disturb the normal functioning of the enzyme.

A simple and convenient description of such a process is provided by Langmuir adsorption equations applied to potassium ions and hydrogen ions competing for the same set of negative centres.

When the displacement of  $K^+$  by  $H^+$  has proceeded far enough, the enzyme will cease to function and the cell to grow. A reasonable approximate formulation of the

condition is that functioning ceases when the fraction of the enzyme surface occupied by  $K^+$  falls below a critical value.

If cells grow and divide in a medium in which the supply of potassium is limited, the sequence of events leading to the cessation of growth may be imagined as follows. The cell population rises, each cell continually producing acid which lowers the pH of the medium. The cells which absorb potassium from the solution to an extent permitting the optimum functioning of the enzyme gradually lose it as the pH falls. As new cells are formed there will be an equal sharing of potassium between them. Eventually the amount available for any given cell falls below the critical limit and further division ceases. In a subsequent stage the further formation of acid by fermentation results in a continued competitive displacement from the negative centres by hydriions, but the cell count remains constant (see appendix).

Let the medium support  $n$  cells. A fraction  $\sigma_K$  of the negative sites on the relevant enzyme is occupied by  $K^+$ , a fraction  $\sigma_H$  by  $H^+$ , when  $[H^+]_{\text{cell fluid}}$  is the hydriion concentration, assumed common to cell and medium, and  $[K^+]_{\text{cell fluid}}$  is the potassium concentration *inside the cell*.

An elementary application of the Langmuir adsorption equations shows that for equilibrium

$$\frac{\sigma_K [H^+]_{\text{cell fluid}}}{\sigma_H [K^+]_{\text{cell fluid}}} = \text{constant}. \quad (4)$$

We have postulated that division ceases when  $\sigma_K$  falls to a constant standard value. Assuming that the enzyme sites are fully occupied with either  $K^+$  or  $H^+$ ,  $\sigma_K = 1 - \sigma_H$ , the condition that growth ceases is

$$\frac{[K^+]_{\text{cell fluid}}}{[H^+]_{\text{cell fluid}}} = \text{constant} = Z. \quad (5)$$

The experiments with radioactive potassium showed that at this stage all but a few per cent of the potassium is inside the cell. If there are  $a$  g. ions in unit volume then there are  $a/n$  in each cell, either in the fluid or on the enzyme surface, so that

$$[K^+]_{\text{cell fluid}} + A\sigma_K = a/n, \quad (6)$$

where  $A$  is a constant.

Combination of (5) and (6) gives

$$Z[H^+]_{\text{cell fluid}} + A\sigma_K = a/n,$$

and if the condition that  $\sigma_K$  has a standard critical value is fulfilled, then

$$\text{constant} [H^+]_{\text{cell fluid}} + \text{constant} = a/n. \quad (7)$$

The final value of  $[H^+]_{\text{cell fluid}}$  corresponding to the actual cessation of growth is difficult to determine. Fermentation goes on producing acid long after growth has ceased, and  $n$  can only be measured after ample time has been allowed to ensure that the population has reached its steady value, the precise moment when growth ceases being unknown.

Equation (7), therefore, is very difficult to test directly. It is best tested in combination with equation (1),  $[H^+]_{\text{cell fluid}}$  being substituted for  $[H^+]_2$ .

The result is a quadratic equation in  $[H^+]_2$ . Without solving this it is obvious, since  $[H^+]_2$  depends only on one variable,  $a/n$ , that

$$\frac{1}{p}[H^+]_1 + \frac{[H^+]_1}{\chi + [H^+]_1} + \frac{1}{p} \frac{k_a}{k} n = f\left(\frac{a}{n}\right). \tag{8}$$

$f(a/n)$  denotes that the right-hand side is, for a given value of  $p$ , a function of  $a/n$  alone.  $[H^+]_1$  is the initial hydron concentration of the medium.

It might have been postulated that the condition for cessation of growth was not a critical value of  $\sigma_K$  but a critical value of the ratio  $\sigma_K/\sigma_H$ . It would then have still followed as before that  $[H^+]_{\text{cell fluid}}$  is a unique function of  $a/n$ .

TABLE 8. INTERPOLATED VALUES OF  $\frac{1}{p}[H]_1 + \frac{[H]_1}{\chi + [H]_1} = F$  AND  $n$  FOR DIFFERENT VALUES OF  $a/n$

$a$ in g.ion/l., $n$ in millions/l. $\chi = 2.0 \times 10^{-7}$ g.ion/l.										
$a/n \times 10^{10} \dots$	2.80		2.60		2.30		2.00		170	
	$F$	$n \times 10^{-3}$	$F$	$n \times 10^{-3}$	$F$	$n \times 10^{-3}$	$F$	$n \times 10^{-3}$	$F$	$n \times 10^{-3}$
0.333	334		0.130	355	0.130	227	0.166	209	0.091	186
0.666	186		0.427	201	0.355	182	0.231	158	0.130	125
0.780	149		0.637	161	0.445	137	0.310	106	0.260	65
0.812	113		0.692	122	0.567	92	0.460	55	—	—
0.875	76		0.780	82	0.677	48	—	—	—	—
—	—		0.833	42	—	—	—	—	—	—

TABLE 9. SLOPES OF LINES IN FIGURE 6

$10^{10}(a/n)$	2.80	2.60	2.30	2.00	1.70
$10^6(k_a/pk)$	2.1	2.3	2.4	2.0	1.5

Mean value of slope = 2.1.

Equation (8) can be tested with the observations on the utilization of potassium at different initial pH values. If a constant value of  $a/n$  is selected and values of  $n$ , obtained from the curves, are plotted against the corresponding values of

$$\frac{1}{p}[H^+]_1 + \frac{[H^+]_1}{\chi + [H^+]_1},$$

then, according to the postulated model, a straight line should be obtained.

Table 8 contains the required values interpolated from the graphs in figure 2, and figure 6 shows to what extent the plots, according to equation (8), are straight and parallel.

A more significant test is provided by the mean absolute value of  $\frac{1}{p} \frac{k_a}{k}$  obtained from these lines, which is  $2.1 \times 10^{-6}$ , the value taken for  $\chi$  being  $2 \times 10^{-7}$  g.ion/l. and the volume unit for  $n$  and  $p$  being the litre.

The slopes of the lines are given in table 9. The mean may be compared with the value obtained from figure 5, which has an initial slope of  $1.5 \times 10^{-6}$ .

The fact that the absolute values are of the same order of magnitude seems to confirm rather definitely that the mechanism assumed is generally correct.

The explanation of why the slopes derived from figure 6 are rather higher than that from figure 5 depends probably upon the excess production of acid during the formation of the last 10 to 20 % of the cells when growth is slowing down. This involves a somewhat greater average value for  $k_a$  in equation (8) than corresponds to the initial slope in figure 5. Provided that the total acid production up to the moment when division ceases remains proportional to  $n$ , linear relations of the type shown in figure 6 will be preserved, even though the slopes are increased.

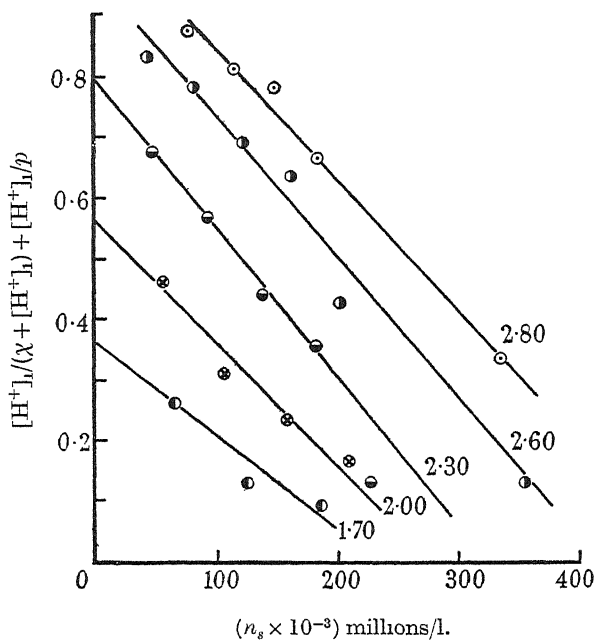


FIGURE 6. Test of equation (8). The numbers on the lines are the values of  $a/n \times 10^{10}$ .

#### ROLE OF POTASSIUM IN GROWTH IN OTHER MEDIA THAN GLUCOSE-AMMONIUM SULPHATE

It is now interesting to examine the potassium requirements of cells growing in media other than the glucose-ammonium sulphate medium so far considered. Any of three possible patterns of behaviour might be expected:

- (a) Growth with certain substrates might be independent of potassium ions.
- (b) Some new phenomenon involving potassium ions might supersede the antagonism between them and hydriions.

(c) An antagonism between potassium and hydriion might still be shown. It might, however, be quantitatively different either because the relative rates of acid production and cell multiplication vary in different substrates, or because the potassium-activated enzyme is a different one or has been modified by adaptation.

In seven different media no example of (a) has been found.

The observations given in figure 7 show the  $n_s$  values for the various added potassium concentrations.

The methods of investigation were as before. Cells were trained by serial subculture in the new medium, buffered to pH 7.10 with phosphate and approximately M/40 in potassium ion. They were then subcultured in similar media lacking added potassium, and buffered by adding hydrochloric acid or sodium hydroxide to sodium phosphate. The sodium salts contained traces of potassium so that limited growth occurred even without added potassium chloride.

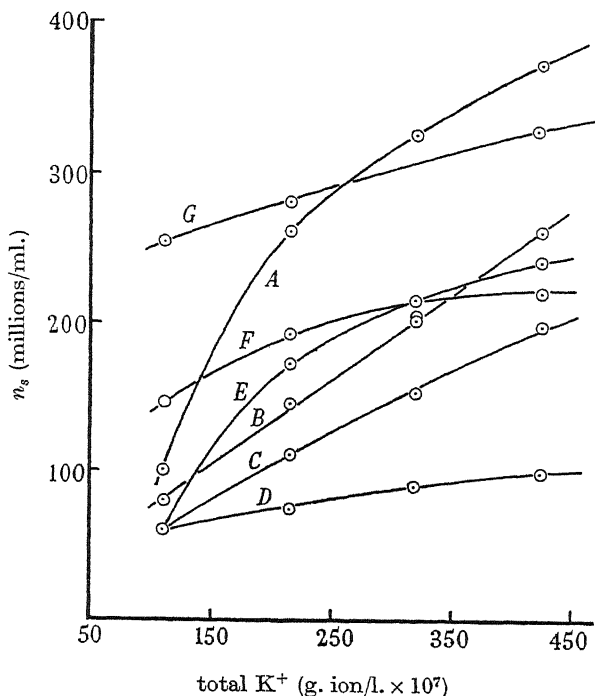


FIGURE 7. Total population and potassium concentration in various media. *Note.* In this diagram the total  $K^+$  concentration is given (i.e. added potassium + that contained in other salts, as estimated by extrapolations from figure 1). *A*, glucose-glutamate; *B*, glucose-ammonia; *C*, glucose-asparagine; *D*, glutamate; *E*, malic acid-ammonia; *F*, pyruvic acid-ammonia; *G*, glycerol-ammonia.

Values of  $n_s$  were measured for graded additions of potassium chloride. The final pH was also determined. Although, as explained above, it may differ appreciably from that prevailing at the point where growth just ceases, it gives at least an approximate indication of the rate of acid production during growth. This rate does in fact vary very widely with different substrates. In some of the media the change of pH is much less than in the glucose medium, while in others there is actually production of alkalinity, mainly from decarboxylation reactions.

Figure 7 shows that the potassium requirement varies widely with the different substrates. Some of the curves are similar in form to that found with the glucose-ammonium sulphate medium, and might be interpretable on the same principles. That for the glycerol-ammonium sulphate medium is of a quite distinct form, and has been investigated in more detail.



## GLYCEROL-AMMONIUM SULPHATE MEDIA

Curve *G* in figure 7 is characterized by a high value of  $n$  in the absence of *added* potassium (though this does not mean zero concentration). On the other hand, additions of potassium beyond the range of concentration shown in the figure raise  $n_s$  steadily to values of nearly 600 million/ml. (table 11). There is no question therefore of the efficacy of the potassium, but the quantities required per cell are for small counts very low and for higher counts become several times larger than with glucose.

The rate of acid accumulation in the glycerol medium was measured and found to be only about one-fourth of that with glucose. Moreover, for a given potassium concentration  $n_s$  does not vary with the initial pH over the range 7.4 to 6.0 (table 10). The three facts, low acid production, high potassium requirement and independence of  $n_s$  and initial pH are not consistent with a competition between hydron and potassium ions during growth.

TABLE 10. TOTAL POPULATIONS IN GLYCEROL MEDIUM

K <sup>+</sup> added (g.ion/l. $\times 10^7$ )	pH ...	$n_s$				
		7.37	7.10	6.74	6.45	6.00
0		212	232	222	240	207
206		286	—	328	363	284
412		345	342	318	360	348
618		348	—	437	377	426

TABLE 11. TOTAL POPULATION AND POTASSIUM CONCENTRATION IN GLYCEROL MEDIUM

Initial pH = 7.10.									
K <sup>+</sup> added (g.ion/l. $\times 10^7$ )	0	153	309	412	772	1030	1545	1802	2060
$n_s$	232	241	314	342	386	470	457	524	551

Nevertheless, potassium can still be regarded as an activator taken up by an enzyme surface. In the present case it seems not to be easily displaceable by hydron, but, it is suggested, by some other product of glycerol metabolism. The amount of this inhibitor is negligible at low counts and then increases, so that the potassium requirement for unit increase of count rises.

It may reasonably be assumed that such an inhibitor is produced at a constant rate by each cell, so that

$$dI/dt = k_i n,$$

where  $I$  is the total amount in unit volume. During logarithmic growth

$$n = n_0 e^{kt},$$

so that  $I = (k_i/k)(n - n_0)$  or approximately  $(k_i/k)n$ . If the fraction of the enzyme surface covered by the inhibitor is  $\sigma_I$ , then from the Langmuir equations we have as before

$$\frac{\sigma_K}{\sigma_I} = \frac{b[K^+]_{\text{cell fluid}}}{I_{\text{cell fluid}}},$$

where  $b$  is constant.

All but a few per cent of the potassium ( $a$  in unit volume) being taken up inside the cells,

$$\sigma_K + A[K^+]_{\text{cell fluid}} = a/n.$$

When growth ceases  $\sigma_K$  must have fallen to the critical value  $\sigma_c$ , which is to be assumed constant. As long as  $n$  is not too small,  $I_{\text{cell fluid}}$  is appreciable, and we may assume that  $\sigma_K + \sigma_I = 1$ . Therefore when growth ceases  $\sigma_K/\sigma_I = \text{constant}$  and consequently

$$\begin{aligned} [K^+]_{\text{cell fluid}} &= \text{const. } I_{\text{cell fluid}} \\ &= \text{const. } n. \end{aligned}$$

Therefore from the above equation the relation between  $a$  and  $n$  assumes the form

$$a/n = \alpha_n + \beta,$$

where  $\alpha$  and  $\beta$  are positive constants.

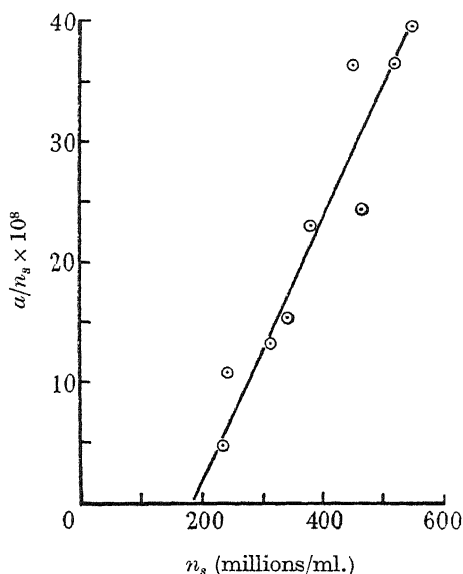


FIGURE 8. Relation of  $a/n$  and  $n$  (glycerol medium).

Figure 8 shows that at higher values of  $n_s$  such a linear relation is approximately obeyed. The line, however, makes an intercept of the wrong sign on the  $n$  axis.

At low values of  $n$  the relation will indeed cease to hold since there is not enough inhibitor in the solution to maintain the condition  $\sigma_K + \sigma_I = 1$ . In these circumstances  $\sigma_K$  will be nearly proportional to  $[K^+]_{\text{cell fluid}}$  so that when  $\sigma_K = \sigma_c$ ,  $a/n$  is constant, and more cells are supported by unit amount of potassium. This factor explains the large intercept in  $G$  of figure 7 for zero added potassium, which represents the growth supported by the potassium in the other salts.

## APPENDIX

*The uptake of sodium and potassium by Bact. lactis aerogenes investigated by means of radioactive tracers*

Radioactive sodium ( $\text{Na}^{24}$ , half-life 14.8 hr.) and potassium ( $\text{K}^{42}$ , half-life 12.4 hr.) were produced by A.E.R.E., Harwell, by neutron irradiation of the carbonates. The samples of sodium and potassium carbonate sent for irradiation were accurately weighed and sealed in small silica tubes. Standard solutions of the active material as chloride could then be made with a minimum of handling by breaking the tube into a beaker containing the calculated quantity of dilute hydrochloric acid and diluting to the required volume.

Activities were measured using 10 ml. samples in a liquid-type Geiger counter. The electronic equipment was of conventional design and was built in the laboratory.

In the first series of experiments inocula were transferred to a synthetic medium containing enough potassium to support a bacterial population of about 300 million cells/ml. Samples were withdrawn at intervals for several hours after the onset of the stationary phase. The cell count was measured and the potassium content of the medium determined from its activity after removal of the bacteria by centrifuging.

TABLE 12. POTASSIUM UPTAKE IN THE STATIONARY PHASE

Glucose-ammonium sulphate medium

age from inoculation (min.)	bacterial population (millions/ml.)	% potassium taken up
388	275	76.6
415	300	69.4
470	300	77.0
525	290	88.7
585	290	94.4
675	290	96.0
765	295	96.7
855	310	97.0
975	300	96.4
1075	300	93.8
1560	310	81.8

Table 12 shows that when growth ceases approximately 97 % of the total potassium in the medium has been taken up, but that during the next few hours some of it is released again. This subsequent displacement is probably due to the acid formed during the continued fermentation of the glucose.

The experiment was repeated with amounts of potassium corresponding to a series of different cell populations. The activity of the medium was measured 12 to 14 hr. after inoculation, at which time the final population is established, but the displacement by acid of the potassium taken up has not yet become serious (table 13).

In the next series of experiments cells were grown to a population of about 300 million/ml. in a medium containing the active potassium. While still dividing they were centrifuged and resuspended in an ammonium phosphate buffer of pH 7.10 containing about half the concentration of active potassium in which they were

originally grown. In this medium they were stirred by aeration for 1 hr. They were then washed again with a non-active phosphate buffer at pH 7.10, and transferred to a series of ammonium phosphate buffers at different hydron concentrations, where they were again stirred for 1 hr. The suspensions were freed of cells by centrifuging and the activity of the supernatant liquid was measured. Table 14 shows that the potassium is progressively displaced from the cells as the pH is lowered.

TABLE 13. POTASSIUM UPTAKE AND BACTERIAL POPULATION

series A initial specific activity of tracer (0.2 mC/g. K <sup>+</sup> )		series B initial specific activity of tracer (2.0 mC/g. K <sup>+</sup> )	
$n_s$ (millions/ml.)	% of K <sup>+</sup> taken up	$n_s$ (millions/ml.)	% of K <sup>+</sup> taken up
130	97.2	35	98.8
170	96.2	48	96.5
177	97.4	48	96.7
195	97.3	62	96.0
227	93.5	62	98.5
265	97.2	72	96.7
310	97.0	72	98.6
365	94.9	105	98.9
		155	99.2
		210	99.4
		245	95.8

TABLE 14. DISPLACEMENT OF POTASSIUM BY HYDROGEN IONS

Cell count of suspension = 190 millions/ml.

pH of buffer	% K <sup>+</sup> displacement from cells into medium
7.10	9
6.64	48
5.66	63
4.30	79

In other experiments cells were washed with non-active isotonic potassium or sodium chloride solution. The suspensions were stirred by aeration for 1 hr., and the activities of the cell-free supernatant liquids were then measured. Tables 15 and 16 summarize these results. It will be seen that ready exchange occurs with potassium, but little displacement is caused by sodium.

TABLE 15. EXCHANGE OF K<sup>42</sup> WITH EXTERNAL NON-ACTIVE ISOTOPE

Initial bacterial population = 180 millions/ml. Successive washings with 40 ml. isotonic potassium chloride (non-active).

washing	activity of 10 ml. cell-free solution (counts/min.)
1	280
2	15
3	3

activity of cell suspension after 3 washings = 0.

TABLE 16. WASHING OF ACTIVE CELLS WITH ISOTONIC SODIUM CHLORIDE

Initial total activity of cell suspension = 330 counts/min./10 ml. Initial bacterial population = 228 millions/ml. Successive washings with 40 ml. isotonic sodium chloride (non-active) of bacteria from 30 ml. original suspensions.

washing	activity of 10 ml. cell-free liquid (counts/min.)
1	9
2	9
3	9

activity of cell suspension (from 10 ml. original) after 3 washings = 296 counts/min.

For experiments on the uptake of active sodium media with two different nitrogen sources, ammonium sulphate and sodium glutamate were employed so that any replacement of sodium by ammonium could be detected.

The sodium content of cells grown in the presence of  $\text{Na}^{24}$  was measured by the activity of a suspension made after the bacteria had been separated in the centrifuge, washed twice with isotonic saline and resuspended in water. Table 17 shows that a negligible amount of active sodium appears in the cells. Since two washings were necessary to remove adhering active solution, the absence of activity in the cells might have been due to exchange between the ordinary isotope and  $\text{Na}^{24+}$ . The use, however, of potassium chloride instead of sodium chloride as washing liquid leads to the same result. It can certainly be said that the cells do not take up any sodium which cannot be replaced by potassium (even when they have had the opportunity of taking up the potassium they require during growth). The low activity of the cells therefore probably indicates that no appreciable amount of sodium was taken up during growth. Even the small residual activity is probably that contained in the traces of washing fluid retained. The absence of sodium in the cells is not due to replacement by ammonium, since the same result is found with sodium glutamate as with an ammonium salt as the nitrogen source.

TABLE 17. UPTAKE OF  $\text{Na}^{24}$

Cells grown in presence of  $\text{Na}^{24}$ : activity of cells obtained from 25 ml. of suspension measured. Potassium phosphate buffer, M/40, used.

ammonium sulphate as nitrogen source		sodium glutamate as nitrogen source	
$[\text{Na}^+]$ (g./ml.)	$\text{Na}^+$ in $10^6$ cells (g. $\times 10^6$ )	$[\text{Na}^+]$ (g./ml.)	$\text{Na}^+$ in $10^6$ cells (g. $\times 10^6$ )
$8.4 \times 10^{-4}$	4.7	$8.4 \times 10^{-4}$	16.0
$8.4 \times 10^{-4}$	2.7	$8.4 \times 10^{-4}$	12.0
$8.4 \times 10^{-5}$	0.3	$8.4 \times 10^{-5}$	2.2
$8.4 \times 10^{-5}$	1.6	$8.4 \times 10^{-5}$	2.2
compare average value for $\text{K}^+$	700	compare average value for $\text{K}^+$	700

Cells grown in non-active medium and subsequently suspended in  $\text{Na}^{24}$ .

$[\text{Na}^+]$ (g./ml.)	$\text{Na}^+$ in $10^6$ cells (g. $\times 10^6$ )
$8.4 \times 10^{-4}$	6.2
$8.4 \times 10^{-5}$	0.3

We should like to thank Mr E. H. Cooke-Yarborough of A.E.R.E., Harwell, for help and advice on the design of the electronic equipment used in this work.

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## An investigation of the nature of certain adaptive changes in bacteria

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Four strains of bacteria which, until trained, show a long lag before they will utilize certain new carbon or nitrogen sources, have been examined by methods involving the transfer of inocula at widely varying dilutions to liquid or solid media containing the new substrate. The strains and sources studied are (a) *Bact. lactis aerogenes* and D-arabinose, (b) *Bact. coli mutabile* and lactose, (c) *Bact. coli* and ammonium sulphate, (d) a *coli-aerogenes* intermediate and sodium citrate.

With (a) and (b) in solid and liquid media and (d) in liquid media all or most of the dilutions which are viable in a glucose-amino-acid medium (which supports growth without lag) also show growth, though after considerable delay, in the new medium. With (c) the proportion of cells which grow is small and, even for parallel inocula, very sensitive to minute changes in the test medium. But in general the reason why many cells fail to grow is found to be that they have died before they have traversed the lag phase which necessarily precedes their development.

The bearing of these observations on theories of mutation and adaptation is considered.

## INTRODUCTION

When certain bacterial strains are transferred from an accustomed medium to one containing a new source of carbon or nitrogen there may be a very prolonged lag phase, after which growth occurs in a more or less normal way. On a second transfer to the new medium there is little lag. The growth rate increases gradually in the course of successive serial subcultures until it reaches a steady optimum value. The same may be true of the total bacterial population which the medium supports. Typical examples of this behaviour are provided by *Bact. coli mutabile* transferred from glucose to lactose (Massini 1907; Postgate & Hinshelwood 1946), *Bact. lactis aerogenes* transferred from glucose to D-arabinose (Jackson & Hinshelwood 1948), *Bact. coli* transferred from amino-acid media to ammonium sulphate as nitrogen

source (Morrison & Hinshelwood 1949) and *coli-aerogenes* intermediates transferred for the first time to media containing sodium citrate as the source of carbon.

Two major hypotheses are possible for the interpretation of these facts. According to the first, mutants are thrown off by chance whenever the strain grows, and some few of these are supposed capable of growing satisfactorily in the new medium. The long lag represents the time required for a minute proportion of the population to multiply up to a size of the same order as the total original number transferred. The fact that the 'trained' strains are sometimes stable and sometimes not is ascribed to the circumstance that 'reverse mutations' may be infrequent or frequent. The circumstance that, after the long lag is entirely eliminated, there is still a progressive improvement of growth rate and of total population on further subculture would presumably be attributed to the occurrence of further secondary mutations upon which selection acts progressively.

According to the second type of hypothesis, the long lag represents the time during which the intermediates necessary for growth by an unaccustomed reaction route are being elaborated by the cell, whose enzymatic mechanisms are initially inadequate for this purpose. Once growth begins, however, the correlation of function and reproduction which must characterize cell systems in general results in an autocatalytic formation of increasing amounts of the enzyme systems concerned in the new reaction sequences. Hence the gradual improvement in growth rate over a series of subcultures. The stability of the modified systems depends upon the modes of interaction between them and any further media in which the cells are grown.

One thing which should be said about these two theories is that they are not necessarily quite so different as might appear at first sight. The first postulates the selection of a few cells favoured by chance, the second envisages the relative increase in certain parts of the internal structure of all the cells, that is, a sort of internal selection. It is not difficult to imagine a whole spectrum of behaviour. At the one extreme there might be modes of cell division occasionally leading to unusual structures endowed with biochemical properties not shared by most. At the other extreme there could be the adaptive modification of every cell in the population. In between there could be statistical variations in the patterns resulting from cell division, so that cells might later undergo the adaptive modifications with different degrees of facility.

It may be artificial to manufacture too clear cut an issue. On the one hand, the existence of statistical variations among cells cannot be questioned. Moreover, certain changes of pattern in the cell material, produced originally by a rather unusual combination of circumstances, can very well be passed on to the progeny of the first cell by the ordinary copying process of autosynthesis. On the other hand, kinetic considerations show that adaptive changes can and must occur. Once these have taken place, they too can affect the progeny in very much the same way. (The dismissal of this possibility as 'Lamarckian' is illogical. The perfectly intelligible reasons why acquired characters are not transmitted in entire animals do not apply to unicellular organisms, where not only the nature of the hereditary process but the very nature of an 'acquired character' is different.) Any change in a bacterium is

itself a change in the germ cell, and its inheritance would be a phenomenon of a different order from that of inheritance of changes in other types of organism.

The first question, then, would seem to reduce itself to one of simple fact: whether in any given example, adaptive changes—using the phrase in a purely descriptive sense—originate in very few exceptional cells, or in a large proportion of them. It would not be wholly surprising if the answer varied from case to case.

Various indirect arguments have been employed in favour of one or other type of hypothesis in particular examples.

By treatment with ultra-violet light, X-rays or certain chemical agents, varieties of the mould *Neurospora* (Tatum & Beadle 1945; Ryan 1946) or of *Bact. coli* (Tatum 1946; Ryan 1946) can be obtained which demand for growth the presence of a certain specific amino-acid. They are termed biochemical mutants. In the case of organisms with a sexual mechanism the enzymatic peculiarities are stated to be inherited according to Mendelian laws, and thus to be located in well-defined genes. This might be held to be more consistent with the mutation theory, though the argument is not by any means conclusive. The biochemical mutants are often unstable and the lost character is readily regained by adaptive training. This process is explained in terms of reverse mutations by some authors (Ryan & Schneider 1948), though the view has also been expressed that in certain cases damaged enzyme systems are repaired when called upon for active use (Peacocke & Hinshelwood 1948). It has been shown that in certain cases gain and loss of properties may occur in conditions where there would be no selection of the adapted and unadapted types, so that the change, whatever its nature, seems to affect the whole population (Cooke & Hinshelwood 1947; Pryce & Hinshelwood 1947).

A great deal of use has been made of a statistical argument. If mutations occur during growth and are inherited, the number of mutants should increase exponentially with time, up to the point where reverse mutations become effective. The mutations being rare and random, there will be a wide scatter in the numbers occurring among the early descendants of a single cell taken as the origin of a strain. This wide scatter will not be eliminated as the progeny of the early mutants multiplies. There might therefore be expected to be very wide differences in the numbers of mutants present in different cultures of the same organism. These numbers can be tested by plating experiments and compared with the divergences found in separate platings from the same culture. There are in fact often found to be very much greater divergences between the different cultures than between the different platings of the same culture. It is argued that adaptive changes induced in large numbers of cells should lead to homogeneous populations so that different cultures should give no more scatter than different individual platings (Luria & Delbrück 1943; Demerec 1945*a, b*; Ryan 1948).

The comparison in question represents, however, a procedure which is very seriously uncontrolled in a most important respect. It is well known that the lag phase of an inoculum transferred to a fresh medium is a quite sensitive function of the age of the parent culture. This factor is of the greatest importance when transfers are made to a medium such as one containing a drug, or one deficient in a normal nutritional constituent, such as is used in tests for mutants. Unless the past history



of the separate cultures which are compared is controlled with an almost impossible degree of accuracy, they will show a scatter due to the uncontrolled ages of the inocula taken from them. This does not apply to parallel platings made from a single culture. The statistical comparison may therefore not be justified. In the case of the separate cultures, their not strictly comparable ages may make all the difference between death and survival in the difficult conditions of a new medium. The maintenance of a constant total time,  $T$ , from inoculation to test of the final culture is a quite inadequate method, since the age of this culture will be  $T - L$ , where  $L$  is the lag. For different cultures  $L$  is known by experience to be quite variable. According to the view that an active adaptation occurs, the picture would be as follows. During the long lag in the new medium many of the cells may die, and in the race between death and adaptation the differences in age become of great significance and give rise to a wide scatter.

Dealing with the mutations of *Bact. coli*, Newcombe (1948) finds that the statistical analysis in terms of mutation rates requires the assumption that there may be a delay in the manifestation in the individuals of the effect of the mutation. Whether, during this time, something like an adaptation occurs is not stated.

A method which has been employed to determine the proportion of mutants in a culture is one (Lewis 1934) which may be illustrated by reference to the lactose variants of *Bact. coli mutabile*.

A strain which has not previously been trained to lactose is plated out on a lactose solid medium, or inoculated at a suitable dilution into a liquid lactose medium. The number of colonies obtained in the first test, or the last dilution at which growth occurs in the second, allows conclusions to be drawn about the fractional number of the original inoculum capable of utilizing lactose. In the experiments of Lewis the fraction was found to be a small, and more or less constant one. The method has this ambiguity: that during the long lag which may have been necessary for growth with utilization of lactose, a large proportion of the cells may have died. It seems necessary to make supplementary tests to determine the actual fate of those cells which fail to grow in the lactose media. Virtanen & Winkler (see Virtanen 1948) found with one strain of *Bact. coli* initially reluctant to use lactose that, although there was a very long lag, ultimately all the cells in the inoculum grew to colonies on a solid medium. In this case, at least, adaptation must have affected the whole population.

The object of the experiments to be described was to investigate further in several typical examples the questions: what proportion of cells would grow in a new liquid medium (or on a new solid medium) as mutants or adapted cells, and what had been the fate, at the moment when the result of the growth test was pronounced negative, of those which had not then grown.

#### EXPERIMENTAL METHODS

Ideally the behaviour of individual cells would be examined but this level of investigation is experimentally almost inaccessible, so that the technique adopted was based upon variations of the well-known dilution methods.

In one of these, successive dilutions, usually increasing by factors of ten, are inoculated into a given medium. The last dilution to show positive growth is assumed to contain a number of viable cells of the order of magnitude unity. In the test for 'mutants' capable of using a new substrate *A* successive dilutions are inoculated in parallel into a standard medium, in which the strain is known to grow without lag under proper conditions, and into the medium *A*. Suppose that the last dilutions to show positive growth in the two cases are  $10^x$  and  $10^y$  respectively, then the number of mutants might be taken to be of the order of magnitude of one in  $10^{x-y}$ .

In the other variation of the test the dilutions are plated on to solid media containing, on the one hand, the standard medium and, on the other, the medium in which the strain is reluctant to grow. The relative numbers of colonies in the two cases can then be compared.

The two modifications of the test have each their advantages and are complementary. In the liquid medium test the *time* required for visible growth has an ambiguous interpretation. It might be that required for an unknown proportion of mutants to multiply up to the threshold of visibility, or it might be a long lag of the majority of the cells. On a solid medium the time for a given colony to appear is more easily interpretable, since the colony is known to have started from a single cell, or at most from a cluster of two or three cells.

The parent culture for a series of tests was diluted in stages of ten in a phosphate buffer of pH = 7.12, 1 ml. of each dilution being successively mixed with 9 ml. of fresh buffer. Small measured inocula of the various diluted suspensions were transferred in parallel to the medium containing the substrate under examination and to a standard medium.

The liquid media were made up as follows:

- 1 ml. magnesium sulphate solution (1 g./l.),
- 10 ml. phosphate buffer (9 g./l.  $\text{KH}_2\text{PO}_4$  adjusted to pH 7.12 with NaOH),
- 10 ml. of a solution of the carbon source,
- 5 ml. of a solution of the nitrogen source.

These last two solutions were (normally) made approximately equivalent in carbon or nitrogen content and contained glucose, 50 g./l.; lactose, 50 g./l.; sodium citrate dihydrate, 50 g./l.; D-arabinose, 5 g./l.; or sodium glutamate, 10 g./l.

Parent strains were normally grown in glucose-ammonium sulphate medium,\* and were used just before the end of the logarithmic growth phase, when the cell

\* Just before transfer to test media the cultures were grown in a solution containing only one-tenth the normal amount of glucose. In these conditions the carbohydrate would be practically completely exhausted before the transfer, and the possibility of significant contamination of the test medium with glucose became very small at all dilutions, and quite negligible at the dilutions of  $10^3$  to  $10^8$  which were used.

This point is of some importance, since it might have been suspected that the cells of the inoculum could multiply at the expense of the glucose carried over, throw off a few mutants in the process and then grow to the threshold of visibility through these latter. In fact, however, even if the residual glucose could have supported 100 % extra growth (an estimate very many times greater than the truth), since it is diluted in the same ratio as the inoculum itself, the amount in the ultimate dilution to give growth in most of the liquid medium tests would only have corresponded to about the amount required for the formation of one extra cell.

population was about  $6 \times 10^8$  per ml. Under these conditions growth began almost immediately from the larger inocula in glucose-ammonium sulphate liquid medium. The parent cultures for tests of the growth of *Bact. coli* in glucose-ammonium sulphate were grown in bouillon in which the cell population was about  $10^9$  per ml.

Solid media were made up in one of two ways, (1) by addition of 2 % agar-agar to a liquid medium, (2) by use of silica gel as the basis, as it was difficult to make later supplies of agar-agar satisfactorily pure. The silica gel media were made by mixing the following solutions and allowing the mixture to set in Petri dishes:

- 10 ml. 10 % water-glass,
- 1 ml. magnesium sulphate solution (1 g./l.),
- 1 ml.  $\text{KH}_2\text{PO}_4$  solution (45 g./l.),
- 1 ml. of a solution of a carbon source (50 g./l.),
- 1 ml. of a solution of a nitrogen source (25 g./l.  $(\text{NH}_4)_2\text{SO}_4$  or 50 g./l. sodium glutamate),
- N HCl to pH 7.1.

Inocula were always exactly equal in volume in the parallel tests. 0.2 ml. inocula, used for the tests in liquid media, were measured by pipette;  $\frac{1}{50}$  ml. inocula, used in most of the tests on solid media, were measured by calibrated Pasteur pipettes, with ends cut square.

For the tests in the liquid medium, tubes were suspended in water-baths at a controlled temperature of  $40.0^\circ \text{C}$ . The solid medium tests were made in an incubator at  $37^\circ \text{C}$ .

#### *Bact. lactis aerogenes and D-arabinose*

Previous experiments (Jackson & Hinshelwood 1948) showed that cells of *Bact. lactis aerogenes* became adapted to use D-arabinose as their source of carbon only after a long lag phase. The duration of this is 20 to 30 hr. when the inoculum consists of  $10^7$  to  $10^8$  active cells, which grow within 2 hr. in 26 ml. glucose-ammonium sulphate liquid medium. The apparent training occurs only during exposure to arabinose while the cells are in a stationary phase. Exposure during growth in glucose does not reduce the length of time which must be spent in the stationary lag phase before growth can occur in D-arabinose.

The long lag might be accounted for either as the time necessary for reorganization of the cells to use D-arabinose for active growth, a process occurring in response to the presence of D-arabinose, or, alternatively, as the time necessary for rare mutant cells, immediately capable of growing in D-arabinose, to increase in number to a figure comparable with the total inoculum, at which point their growth would be detectable.

It has frequently been suggested that bacterial mutations occur with a frequency of about  $10^{-6}$ . We may therefore consider the implications of the possible presence of 100 mutant cells in an inoculum of  $10^8$  cells. Suppose they are all immediately capable of using D-arabinose for growth. They would be supposed to increase in number to  $10^8$  in about 25 hr., the mean generation time being thus about 75 min. This is in fact the mean generation time actually observed for growth in D-arabinose when growth is well established. Other observations with a strain of *Bact. lactis aerogenes* which required a lag of 60 hr. before detectable growth in D-arabinose

(mean generation time 75 min.) would be explicable if the earlier, undetectable growth were slower. One strain has in fact shown a mean generation time of about 400 min. after a lag of 160 hr.

So far, then, either of the theories could account for the experimental observations. The dilution tests on solid and liquid media were therefore applied.

### *Experiments with solid media*

The solid media were of the same composition as the liquid media, with the addition of 2 % agar-agar. Inocula of 0.1 ml. were used, the drops being dispersed over the surface of the plates by spinning. Liquid medium controls were used as a check on the activity of the cells. Inocula of  $10^8$  cells in glucose-ammonium sulphate liquid media showed growth within 2 hr. in all the experiments.

The results are collected in table 1.

TABLE 1. *BACT. LACTIS AEROGENES* AND D-ARABINOSE OR GLUCOSE.  
SOLID MEDIUM

dilution	number of colonies on glucose plate	time before appearance of colonies on glucose (hr.)	number of colonies on D-arabinose plate	time before appearance of colonies on D-arabinose (hr.)	lag in D-arabinose liquid medium (hr.)
$10^6$	36	17	20	31-41	31
$10^6$	37	17	26	31-41	31
$10^7$	5	17	2	31-41	31
$10^7$	6	17	7	31-41	31
$10^6$	56	6	90	33	22
$10^6$	44	6	70	33	22
$10^7$	1	6	6	33	22
$10^7$	1	6	2	33	22
$10^7$	11	14	3	33	23
$10^7$	8	14	3	33	23

The strain under study does not clump and each colony springs from a single cell, or at most a very small cluster of cells. In all experiments, similar numbers of colonies grew from parallel inocula on glucose and D-arabinose plates. None appeared on plates containing no added source of carbon, within the duration of an experiment (1 week).

Clearly, all the cells of an inoculum which are capable of growth in glucose are also capable of growth in D-arabinose. The appearance of visible colonies on glucose plates began within 17 hr. of inoculation at most; on D-arabinose the colonies appeared after about 30 hr. In any one experiment they appeared at very nearly the same time. A similar lag was observed in the D-arabinose liquid medium. This shows that most of the individual cells are able to reorganize to permit growth in D-arabinose in approximately the same length of time.

In this example of adaptation there seems to be little question of selection of special mutants. All the individual cells appear to be rather similar in their ability to undergo adaptation in response to the presence of D-arabinose.

*Experiments with liquid media*

The above results are confirmed by dilution experiments in liquid media. All inocula which have a reasonable chance of containing one cell grow quickly in glucose media and after a long lag in D-arabinose media. Smaller inocula do not grow. The lags increase with dilution, as might be expected in view of the dilution of diffusible intermediate metabolites.

A typical set of results is recorded in table 2.

TABLE 2. *BACT. LACTIS AEROGENES* AND D-ARABINOSE OR GLUCOSE.  
LIQUID MEDIUM

lags calculated assuming mean generation times 30 min. (glucose) and 75 min. (D-arabinose)

dilution	approx. number of cells in inoculum	growth in glucose	approx. lag in glucose (hr.)	growth in D-arabinose	approx. lag in D-arabinose (hr.)
1	10 <sup>8</sup>	+	< 10	+	29
10	10 <sup>7</sup>	+	< 9	+	28
10 <sup>2</sup>	10 <sup>6</sup>	+	< 7	+	26
10 <sup>3</sup>	10 <sup>5</sup>	+	< 6	+	24
10 <sup>4</sup>	10 <sup>4</sup>	+	< 4	+	48
10 <sup>5</sup>	10 <sup>3</sup>	+	4	+	49
10 <sup>6</sup>	10 <sup>2</sup>	+	< 24	+	41
10 <sup>7</sup>	10	+	< 22	+	56
10 <sup>8</sup>	1	+	< 20	+	55
10 <sup>9</sup>	< 1	—	> 8 days	—	> 8 days
higher dilutions	< 1	—	> 8 days	—	> 8 days

Further confirmation of the nature of the adaptations of *Bact. lactis aerogenes* to D-arabinose has come from another approach. P. C. Caldwell, in this laboratory, has shown that, during the lag phase of this bacterium in D-arabinose, the organic phosphate content of the cells remains steady until just before detectable division begins. It then increases rapidly in a way which seems only explicable by a change in at least the majority of the cells present. This phenomenon is typical of the end of a true lag phase, when the majority of cells are about to divide.

*Bact. coli mutabile and lactose*

This system has been investigated by several authors and especially Lewis (1934). For the present investigation the methods were similar to those used for the experiments with *Bact. lactis aerogenes* and D-arabinose, with two necessary modifications.

*Bact. lactis aerogenes* readily uses ammonium sulphate as a source of nitrogen. *Bact. coli mutabile* is somewhat reluctant to do this, however thoroughly it is trained. The strain examined, when trained to the glucose-ammonium sulphate medium, would only grow if inocula of not less than 10<sup>7</sup> cells were used. This medium was therefore unsuitable for testing the growth of small inocula, and sodium glutamate was substituted as the source of nitrogen for control cultures.

A second difficulty was to find a suitable base for solid medium experiments. The agar-agar available was able to support some growth of colonies without the addition of any other nutrients. Silica gel media, made up as described, were therefore used.

*Experiments with solid media*

The results of a typical experiment are summarized in table 3. Inocula of  $\frac{1}{50}$  ml. were used at each dilution. Standard inocula of  $10^8$  cells grew immediately in glucose liquid medium, and after 60 hr. (the typical behaviour for this strain) in lactose liquid medium.

TABLE 3. *BACT. COLI MUTABILE* AND LACTOSE OR GLUTAMATE.  
SOLID MEDIUM

dilution	number of colonies on glutamate	time before appearance of colonies on glutamate (hr.)	number of colonies on lactose	time before appearance of colonies on lactose (hr.)	approximate number of cells in inoculum
$10^4$	c. 1000	< 14	c. 1000	43-62	4000
	c. 1000	< 14	c. 1000	43-62	—
$10^5$	110	< 14	100	43-62	400
	106	< 14	204	43-62	—
$10^6$	13	< 14	14	43-62	40
	10	< 14	30	43-62	—
$10^7$	0	> 5 days	0	> 5 days	4
	0	> 5 days	7	43-62	—

The glutamate-silica gel media were checked against broth-agar media. Parallel inocula yielded the same number of colonies.

In some experiments a few very small colonies appeared after 5 days on control plates containing no source of carbon. This effect could not have interfered with the main experiment, growth on lactose media being complete in 62 hr., when the colonies were much larger and more numerous.

This result closely parallels that obtained for the growth of *Bact. lactis aerogenes* in D-arabinose.

It seems clear that with this strain all cells capable of growth on sodium glutamate are also capable of growth on lactose-ammonium sulphate solid media after the requisite lag phase. The result corresponds to that described by Virtanen (1948).

*Experiments with liquid media*

Although very small inocula grow reliably on solid lactose-ammonium sulphate media, they often failed to do so in liquid media of similar composition.

The results of a typical experiment with liquid media are given in table 4.

The two smallest inocula which grew in sodium glutamate did not grow in the lactose-ammonium sulphate medium. Here there is an apparent difference in the numbers of the original cells which are capable of using lactose.

But the matter is not to be dismissed in this way. When sodium glutamate was now added to the tests, after 6 days, there was still no growth. It thus appears that

TABLE 4. *BACT. COLI MUTABILE* AND LACTOSE OR GLUTAMATE.  
LIQUID MEDIUM

dilution	approx. number of cells in inoculum	lag in glutamate medium (hr.)	lag in lactose (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> medium (hr.)
1	10 <sup>8</sup>	< 4	82
10	10 <sup>7</sup>	< 3	85
10 <sup>2</sup>	10 <sup>6</sup>	1	103
10 <sup>3</sup>	10 <sup>5</sup>	< 14	116
10 <sup>4</sup>	10 <sup>4</sup>	< 12	113
10 <sup>5</sup>	10 <sup>3</sup>	< 10	110
10 <sup>6</sup>	10 <sup>2</sup>	< 8	94
10 <sup>7</sup>	10	< 6	> 6 days
10 <sup>8</sup>	1	< 6	> 6 days
higher dilutions	—	> 6 days	> 6 days

the cells at the higher dilutions had all died before they could grow on the lactose and before the glutamate was added.

In the experiments of Lewis the strain of *Bact. coli mutabile* did not grow on lactose until 5 to 8 days had elapsed, when very few colonies appeared compared with the number which appeared almost at once on broth-agar plates inoculated in parallel. The question now arises whether in fact most of the cells had not died during the 5 to 8 days, and whether, had they lived they would not have become adapted to lactose in the same way as those which did survive.

#### *Bact. coli and ammonium sulphate as nitrogen source*

The experimental methods were similar to those described in the previous sections, except that the parent culture was grown in broth. Inocula of 10<sup>8</sup> cells grew almost immediately in glucose-sodium glutamate liquid medium, and after 50 hr. in glucose-ammonium sulphate.

#### *Experiments with solid media*

Very large differences in behaviour were found for identical inocula on glucose-ammonium sulphate, although the strain behaved normally on glucose-sodium glutamate and on broth agar.

Table 5*a* shows the results of typical experiments with the latter media, and tables 5*b* and *c* in contrast shows the variable results from parallel inoculations on to glucose-ammonium sulphate plates.

Table 5*c* gives the history of development of the colonies on seven different plates, each inoculated with four well separated drops from a 10<sup>8</sup> dilution.

In some cases it seems clear that the growth of a few colonies provided diffusible growth factors which made possible the development of others. Possibly the results for 71 and 171 hr. respectively give an approximate idea of the unaided and the aided growth.

The variations in the behaviour of the different inocula from the same dilution evidently depends upon subtle changes outside experimental control. These will

include minute variations in the amount of intermediate metabolites carried over with the inoculum.

Cells which do not develop the ability to utilize ammonium sulphate die off fairly rapidly. When drops of sodium glutamate were added in the vicinity of the inoculation sites on plate IV after 114 hr. only nine colonies appeared in the three zones which had not previously yielded any. Similar tests on plates II and VI after 71 hr. gave respectively no colonies and confluent growth, a result showing that in one case the cells had all died and in the other not.

TABLE 5*a*. *BACT. COLI*: GLUCOSE-SODIUM GLUTAMATE AND BROTH-AGAR MEDIUM

dilution	number of colonies on glucose-sodium glutamate	time before appearance of colonies on glucose-sodium glutamate (hr.)	number of colonies on broth agar	time before appearance of colonies on broth agar (hr.)
10 <sup>6</sup>	38	28	24	5-17
	28	28	23	5-17
	40	28	31	5-17
	35	28	26	5-17
	46	28	30	5-17
	42	28	30	5-17
	53	28	31	5-17
	37	28	30	5-17
10 <sup>8</sup>	∞	5-17 hr.	∞	< 5 hr.

(The symbol ∞ represents confluent growth of colonies within the inoculum site of a  $\frac{1}{50}$  ml. inoculum. From such an inoculum, growth occurs within a circular area of about 1 sq.cm. On the ammonium sulphate plates, confluence usually occurs by expansion of existing colonies. In the other plates it is due to simultaneous appearance of a large number of colonies.)

TABLE 5*b*. *BACT. COLI*: GROWTH OF 10<sup>6</sup> DILUTION ON GLUCOSE-AMMONIUM SULPHATE PLATES

time (hr.)	number of colonies						
	plate I	II	III	IV	V	VI	VII
65	0	0	0	0	0	0	0
71	0	0	0	0	0	0	2
89	0	0	0	0	0	0	8
100	0	0	7	0	0	0	8
171	0	0	7	0	0	0	8

In these experiments it is evident that there are considerable differences in the potentialities of individual cells to develop the ability to use ammonium sulphate. Even with large inocula a long-lag phase is usually necessary. Some cells develop much earlier than others which may die. A good deal of the variation must be ascribed to the chance of survival, and there is no reason to suppose that the less fortunate cells would not overcome their handicap after a further period were they able to survive so long. Indeed, it appears that they only require slight assistance to achieve the change necessary for growth.



TABLE 5c. *BACT. COLI* AND AMMONIUM SULPHATE  $10^3$  DILUTION

time (hr.)	plate I				plate II				plate III				plate IV			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
41	2	.	.	.	.	.	.	.	∞	20	10	.	1	.	.	.
53	10	10	10	.	.	.	.	.	.	100	100	.	4	.	.	.
65	∞	.	.	.	.	.	.	.	.	.	.	.	14	.	.	.
71	∞	50	20	3	0	0	0	0	∞	100	100	2	40	0	0	0
	Na glutamate added															
89	.	∞	100	13	.	.	.	.	.	∞	∞	30	.	.	.	.
100	.	.	.	20	.	.	.	.	.	.	.	.	100	.	.	.
114	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.
	Na glutamate added															
137	.	.	.	50	.	.	.	.	.	.	.	.	.	6	2	.
171	∞	∞	100	50	1	0	0	0	∞	∞	∞	30	∞	6	2	1

time (hr.)	plate V				plate VI				plate VII			
	1	2	3	4	1	2	3	4	1	2	3	4
22	.	.	.	.	.	.	.	.	∞	∞	∞	∞
41	1	.	.	.	1	.	.	.	.	.	.	.
53	8	.	.	.	.	.	.	.	.	.	.	.
65	20	.	.	.	5	.	.	.	.	.	.	.
71	50	0	0	0	5	0	0	0	∞	∞	∞	∞
	Na glutamate added											
89	.	2	.	.	∞	∞	∞	∞	.	.	.	.
100	100	50	7	4	.	.	.	.	.	.	.	.
114	200	100	50	5	.	.	.	.	.	.	.	.
137	∞	∞	∞	200	.	.	.	.	.	.	.	.
171	∞	∞	∞	200	∞	∞	∞	∞	∞	∞	∞	∞

Note. For any given plate the numbers 1, 2, 3, 4 indicate the separate inoculation sites.

For any given inoculum the first figure recorded in a vertical column is the number of colonies present on the first occasion that growth was observed. Blanks in the same column for earlier times indicate no growth, for later times that there was no further important change. The columns are filled in completely for 71 and 171 hours.

### Experiments with liquid media

These were made as before. In glucose-sodium glutamate controls growth occurred without serious delay down to dilutions of  $10^7$  or  $10^8$ . Higher dilutions failed as expected.

In the glucose-ammonium sulphate medium growth would only occur with the largest inoculum. Dilutions from  $10^3$  to  $10^8$  all failed.

After 5 days sodium glutamate was added to the liquid medium in all the tests which had so far failed to show growth, in order that any viable cell might now develop. In most of the tubes there was no development, a result showing that most of the cells had died before the end of the test (table 6).

### *A coli-aerogenes intermediate and sodium citrate as carbon source*

The strain was isolated by several platings. It grew in the normal media and used citrate as a source of carbon after a delay of about 12 hr. in conditions where the lag in a glucose medium would have been zero. The mean generation time in citrate

TABLE 6. *BACT. COLI* AND AMMONIUM SULPHATE. LIQUID MEDIUM

dilution	approx. number of cells in inoculum	growth in glutamate	lag in glutamate (hr.)	growth in ammonium sulphate	lag in ammonium sulphate	growth on addition of glutamate to ammonium sulphate test	
						5 days	7 days
1	10 <sup>8</sup>	+	5-12	+	62 hr.	+	+
10	10 <sup>7</sup>	+	2-9	-	> 7 days	+	-
10 <sup>2</sup>	10 <sup>6</sup>	+	< 6	-	> 7 days	-	-
10 <sup>3</sup>	10 <sup>5</sup>	+	< 3	-	> 7 days	-	-
10 <sup>4</sup>	10 <sup>4</sup>	+	3	-	> 7 days	-	-
10 <sup>5</sup>	10 <sup>3</sup>	+	11	-	> 7 days	-	-
10 <sup>6</sup>	10 <sup>2</sup>	+	4	-	> 7 days	-	-
10 <sup>7</sup>	10	+	< 12	-	> 7 days	-	-
10 <sup>8</sup>	1	+	< 9	-	> 7 days	-	-
higher dilutions	0	-	> 14 days	-	> 7 days	-	-

being about 1 hr., the proportion of 'citrate mutants' present would have to be one in 10<sup>3</sup> to 10<sup>4</sup> to explain the delay. If this were so, in a liquid medium test giving positive growth down to 10<sup>8</sup> dilution with glucose the citrate media should give negative results after 10<sup>4</sup> or 10<sup>5</sup> dilutions.

#### *Experiments with solid media*

Growth on the citrate-silica gel medium was irregular and erratic. The investigation was not pursued, since experiments with liquid media gave quite definite results.

#### *Experiments with liquid media*

There was exact correspondence at all dilutions between the results with glucose and those with citrate media. All inocula which grew in glucose also grew in citrate after the appropriate delay, as shown in table 7.

TABLE 7. *COLI-AEROGENES* INTERMEDIATE AND CITRATE. LIQUID MEDIUM

dilution	approx. number of cells in inoculum	growth in glucose medium	lag in glucose medium (hr.)	growth in citrate medium	lag in sodium citrate medium (hr.)
1	10 <sup>8</sup>	+	1	+	13
10	10 <sup>7</sup>	+	1.5	+	11.5
10 <sup>2</sup>	10 <sup>6</sup>	+	4-7	+	8
10 <sup>3</sup>	10 <sup>5</sup>	+	14	+	23
10 <sup>4</sup>	10 <sup>4</sup>	+	7	+	30-48
10 <sup>5</sup>	10 <sup>3</sup>	+	11.5	+	55
10 <sup>6</sup>	10 <sup>2</sup>	+	10.5	+	45
10 <sup>7</sup>	10	+	12-23	+	53
10 <sup>8</sup>	1	+	10-21	+	67
higher dilutions	—	-	> 5 days	-	> 5 days

## DISCUSSION

From the study of these various systems in which bacteria grow in a new substrate only after a long delay, it has emerged that all cells are ultimately able to develop provided that they survive the long lag phase. In those examples where all grow there is no evidence of a selection of special cells: in those cases where only a few develop, it seems primarily their survival capacity which varies.

The well-known approximately exponential decay curve which expresses the death rate of members of a large bacterial population shows in fact that there are very wide variations in survival time in every kind of culture. Whether or not the adaptive adjustments occurring during the lag phase will have completed themselves before a given cell dies will, in difficult circumstances, therefore, be subject to great uncertainty.

In the study of the *Bact. coli*-ammonium sulphate system there have been found consistently very wide fluctuations in the growth of apparently identical inocula taken from the same parent culture at the same time, although the same experimental technique with other systems gave reproducible results. This we attribute to the fact that the chances of survival or death during the lag phase are about equal, and that the former are sensitive to minute differences in the amounts of intermediates carried over in the inoculum, possibly inside the cells themselves.

This point of view is different from that which has not infrequently been expressed (see introduction). Some investigators have supported the hypothesis of previous mutation by comparing the variations in the growth of different cultures with those in the growth of parallel inocula from the same culture. These experiments, as has been pointed out, are subject to the uncertainty that the inocula from different cultures cannot possibly be as well controlled in respect of age as inocula from the same culture. In the present experiments very wide variations occur among identical inocula as soon as the conditions of survival become difficult, and we attribute this to statistical rather than to inherited fluctuations.

In those examples where all the cells grow after a delay it must be pointed out further that there is none which appears to grow without lag, as the normal form of mutation theory would demand. It might of course be said that the mutation rate is so great that all the cells present mutate during the course of the experiment. This may be so, but such a point of view involves the use of the term mutation in a sense rather profoundly different from that normally accepted. Possibly, however, some of the divergences of opinion in this matter may, as has happened before, be ultimately reduced to a question of lexicography.

In any case hypotheses in terms of chemical kinetics may be constructed to suggest how adaptive changes can in fact occur, and, if we wish to speak of a whole cell population as undergoing a mutation, it does not seem unreasonable to propose the chemical hypothesis as the interpretation of what this mutation involves.

*Note added in proof, 8 September 1949*

Since this paper was sent in the valuable study of F. J. Ryan & L. K. Schneider (1949, *Genetics*, **34**, 72) has reached us. These authors describe cases somewhat similar to those of the present paper. They use strains of *Bact. coli* which have become

biochemically exacting, and they conclude that delayed growth of these depends upon traces of impurities in media which support the growth of micro-colonies or subvisible growth (in liquid media) during which mutations occur. Rather specific assumptions about the impurities present have to be made: the glucose must be assumed to contain lysine, methionine, leucine, arginine(?) and proline, but to be free from histidine and probably threonine, and whether or not a micro-colony grows to a visible one must be assumed to depend upon rather ill-defined conditions of crowding. Ryan & Schneider, however, also report growth of an arginine-dependent strain without the prior formation of the micro-colonies.

The considerations which they advance are very important, but in the present case the *ad hoc* assumption of appropriate amounts of impurities would not provide a satisfactory explanation. With inocula of  $10^8$  cells we find a lag in D-arabinose of 30 hr. Even if there were enough impurity present to support heavy visible growth which doubled this number of cells, the number of mutants would only thereby be doubled also, and the change in apparent lag which resulted would be negligible. To account for the actual lag we still have to assume about one mutant in  $10^6$  in the original strain. In a solid medium test with an inoculum of a few cells only, growth supported by impurities would have to occur to an extent of well over  $10^6$  cells before mutants appeared, and the colonies thereby formed would already be clearly visible and outside the limits ( $10^4$  to  $10^5$ ) which Ryan & Schneider call micro-colonies. Such colonies would by hypothesis appear without lag.

The application of the interpretation of Ryan & Schneider to the present results meets with other difficulties in connexion with rate of reversion from the adapted to the unadapted state. These will be dealt with in a subsequent communication.

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# The effects of D-xyloketose and certain root exudates in extension growth

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This investigation has been designed to examine the possibility that the substance which is exuded from the roots of *Sorghum* seedlings, and which stimulates the germination of the seeds of *Striga hermonthica*, may promote extension growth in the roots of a variety of species. The technique used involves observations on fragments 1.5 mm. long excised from the extending zones of roots of peas and maize. The effects on the growth of these segments of certain solutions of the natural exudate and of D-xyloketose, which also stimulates the germination of *Striga* seeds in very low concentrations, have been examined. It has been found that the exudates and the sugar promote extension growth in the root by stimulating longitudinal and lateral extension. Moreover, it has been shown that as with stimulation of the germination of *Striga* seeds, the activity of exudate and sugar solutions is destroyed by heat and it decreases during storage of the solutions. The conclusion is drawn that the substance which is released from the roots of *Sorghum* seedlings is a generalized root extension stimulator which promotes the germination of *Striga* seeds. The substance may be a carbohydrate or a simple derivative and may bear a close relationship to D-xyloketose.

## INTRODUCTION

The present series of observations is a development from an investigation of the nature of the stimulant involved in the germination of *Striga hermonthica* (Brown, Johnson, Robinson & Todd 1949). It was shown that the stimulant which is released from a potential host root may be a simple carbohydrate, and that germination may be induced with D-xyloketose in lower concentrations than it is with the purest preparations of the natural stimulant that have so far been made. Earlier, Brown (1946) had suggested that the natural substance may be one that is involved in the growth of the roots of a large variety of species, and the results given with D-xyloketose in the germination of *Striga* provided the opportunity for examining this possibility. The present inquiry was designed to determine the effect of the natural root exudate and of D-xyloketose on the extension growth of peas and maize. The investigation has been restricted to extension growth, since cytological observations on the effect of the stimulant on the germination of the *Striga* seed had shown that the immediate effect involves the extension of the cells of the radicle. In the early stages of germination there is no division in the apex of the radicle, and this only begins when the organ has reached a considerable length.

Observations have not been made on intact roots, but on fragments taken from the extending zone. Since the stimulator is released from actively growing roots it is possible that addition of this substance to the medium in which roots are

growing would not have any effect. It might have an effect, however, if the extending zone is isolated from possible sources of supply in the meristematic region on one side and in mature tissue on the other. At the time when the investigation was started segments excised from the extending zone were being used in the Leeds laboratory for general studies on extension growth in the root. It had been shown that growth in the segments is by extension only, and accordingly the early observations with the exudate and with the sugar were made with them. Since the preliminary experiments showed that the segments reacted to both the exudate and the sugar all subsequent work has been done with these fragments, the use of which has the further advantages that it enables the results of a single experiment to be obtained within 18 hr., and with a single application of an experimental solution. These considerations are of some importance since the solutions are always highly unstable, and the activities of different preparations inevitably vary considerably.

The results presented below show that both natural root exudates and D-xyloketose have marked stimulating effects on extension growth in the root at appropriate concentrations. They also show, however, that the stimulation involves both longitudinal and lateral extension. In absolute terms the increase in breadth due to the stimulants is not great (in favourable circumstances the diameter of the sugar-treated tissues may be 10 % greater than that of the water controls), but since a slight increase in diameter implies a relatively greater increase in volume, this aspect of the position has been examined in some detail, and measurements of breadth as well as of length have been made in all experiments.

It may be emphasized here that while the present series of experiments have shown that root exudates and D-xyloketose stimulate extension growth in the root in concentrations characteristic of hormone action, they have not shown that sugars do not exert stimulatory effects in organs other than the root and in processes other than extension.

A preliminary note on the results of this investigation has been published elsewhere (Brown, Robinson & Johnson 1949).

## METHODS

The methods and techniques of the investigation are described below under four headings: (1) characteristics of the segments, (2) preparation and culture of the segments, (3) preparation and treatment of the experimental solutions, and (4) general experimental design. Finally, we include (5) a short statement on the analysis and presentation of results.

### *Characteristics of the segments*

As indicated above the properties of different fluids have been examined with segments excised from the extending zones of roots of peas and maize. The fragments are each 1.5 mm. in length, and they occupy in the parent roots the region extending from 1.5 to 3.0 mm. from the tip. It has been found that when supplied with an inorganic salt mixture dissolved in 2 % sucrose the excised segments of peas may reach a length of about 6 mm. after 24 hr. The increases that have been measured

in this investigation are much less than this, but the observations with the complete medium indicate that they have been obtained with a tissue which is potentially capable of normal extension growth.

At the time of excision the segments, while they consist almost entirely of cells which have already vacuolated, also contain sporadic non-vacuolated cells. It has been shown, however, that in the conditions of these experiments cell division does not occur and that the total number of cells does not change during the experiment. The absence of cell division has been shown by direct microscopic examination of the tissue after staining with acetocarmine, and the constancy of the total number of cells by using the maceration technique described by Brown & Rickless (1949).

#### *Preparation and culturing of the segments*

The segments are excised from roots obtained from seeds germinated at 25° C in the dark on sand with constant moisture content. When the roots are 3 to 4 cm. long they are detached from the seeds and placed in batches of ten in a cutter which excises the appropriate zone from the extending region of each root. It may be emphasized that the conditions in which the seeds are germinated influence profoundly the reactions of the segments to experimental treatment. We have found that large differences between control and experimental series are given only when the seeds are maintained rigidly in the dark and when the water content of the sand is such that the roots are not waterlogged when they emerge from the seeds.

After excision the segments are transferred to sintered glass disks standing in experimental fluids in Petri dishes. When in position the upper edge of each disk is slightly above the level of the liquid. The upper surface of the disk is moist, and the segments are therefore in direct contact with the fluid without being immersed in it. In these circumstances the gaseous exchange of the tissue is relatively unrestricted.

Twenty segments are allocated to each disk, and each culture is incubated at 25° C for 18 hr. in the dark. At the end of this time the length and breadth of each segment is measured microscopically against a calibrated micrometer eyepiece using a 2 in. objective.

#### *Preparation of the experimental solutions*

Brown & Edwards (1944) showed that seeds of *Striga* germinate when they are irrigated with water in which the roots of *Sorghum* seedlings had been growing. Also that when ten seedlings are grown with 20 ml. of water at 25° C a solution of the stimulant of high and constant activity is obtained. A solution prepared in this way has been used in the present series of experiments, and is referred to below as the standard solution.

A second preparation of the natural stimulant has also been used obtained by growing roots of seedlings of *Sorghum* in water containing charcoal, on to which the stimulant is adsorbed, and from which it is subsequently recovered by eluting with 70 % acetone. The preparation of this material has been described by Brown, Johnson, Robinson & Todd (1949). The eluate after separation from the acetone is redissolved in distilled water, and this solution is referred to as that of the concentrate.

The D-xyloketose used has been prepared in two ways, biologically from D-arabitol (Prince & Reichstein 1937), and chemically from D-xylose (Levene & Tipson 1936).

Initially the concentrate and the sugar are dissolved in glass distilled water at the rate of 1.0 g/l., and in each case this constitutes the basic solution from which series of dilutions are prepared and to which various treatments are applied.

#### *General experimental design*

The growth-promoting properties of any particular preparation are examined with six solutions, of which each member of the series is a tenth of the concentration of the preceding one. The dilutions are made from the crude standard solution, and from the basic solutions (1.0 g./l.) of the concentrate and of the sugar. The total solute concentrations in the solutions of the standard are of course not known; but the solutions of the other two preparations give a series containing respectively 1000, 100, 10, 1.0, 0.1 and 0.01 mg. of the solute per litre. In each case the effects given with the series of six experimental solutions are compared against those given with water in a control culture, and a complete test therefore involves seven cultures. Typically six dilutions are used, but in certain instances for various reasons the number has been reduced to five, and the test in these instances comprises six cultures.

It has been shown that in the stimulation of the germination of the *Striga* seed D-xyloketose shows many of the properties of the natural exudate. Solutions of the natural substance and of the sugar are highly unstable; the activity of both falls rapidly during storage and they are both rapidly inactivated by heat. Accordingly, in the present connexion the effects have been examined of refrigerator storage at 3° C and of heating at 96° C for 90 min. The treatments are in each case applied to the basic solution. With storage treatment after making a series of dilutions from an aliquot of a fresh standard solution or basic solution, the rest of the sample is placed in the refrigerator for 24 hr., after which a further series of dilutions is made, and again after a further period of storage of the remainder for 24 hr. Similarly, after making the usual series of dilutions from an aliquot of a fresh standard or basic solution, the effect of heat treatment is determined by placing the remainder in a boiling tube in a water-bath and after the appropriate time, cooling it and then making another series of solutions from the treated material.

In the normal procedure one Petri dish with a single disk carrying a set of twenty segments is used for each dilution. A single experiment therefore involves seven dishes and 140 segments when six dilutions are used and six dishes and 120 segments when five only are used. As indicated above each segment is measured separately, and each dish therefore yields twenty observations each of length and breadth. A mean is calculated from each set of twenty measurements, and this is taken to represent the result given by the single dish. In the next section the primary data quoted are all mean values.

Most of the results given below have been obtained with pea-root segments, and the nature of the biological material is only indicated when it is not pea-root segments.



*Presentation and analysis of results*

In the next section in addition to the primary data two derivative sets of values are given. From the mean lengths and breadths the average volume has been calculated, and from this again the percentage increase in volume, which represents the increase in volume expressed as a percentage of the volume at the beginning of the experiment. The initial volume is derived from the known standard length which is 1.5 mm. and from the measurements of the breadths of twenty segments which are made after these are excised and before they are transferred to one of the experimental dishes.

The mean initial breadth of the segments used in different experiments varied slightly and the value (indicated by IB) recorded for each experiment is given with each set of results.

The set of data from each experiment has been examined statistically and the significance of the differences within any set of six or seven means have been determined. The total variance for each group of 140 or 120 observations has been calculated and assigned to treatment and error, 133 or 114 degrees of freedom being allowed for error and 6 or 5 for treatment. The significance of the differences has been assessed from the variance ratio. In each table of the next section the significance of the differences is denoted by NS, S or HS which indicate respectively not significant, significant at the 5 % level, and significant at the 1 % level.

It is evident from the array of data given below that the results obtained with water and with very dilute solutions (which may be considered as replicate controls) for the same occasion agree closely. On the other hand, the results given by water controls for different occasions differ widely, and it is therefore more difficult to compare the results for different occasions than those for the same occasion. This difficulty affects the interpretation of the results of the storage experiments, since these involve serial observations on successive days. The variation in the results given with the controls on different days has apparently been due to slight differences in the moisture content of the sand in which the seeds have been germinated.

## RESULTS

The results are presented in three groups in relation separately to the effects of the standard solution, of the concentrate, and of D-xyloketose.

*Standard solution*

The effect of an untreated fresh preparation of the standard solution is shown by the relevant data of tables 1 and 2 and by the curves of figure 1, which are constructed from the values for the untreated solution given in table 1. Evidently the standard solution has a marked stimulating effect, especially when the concentration has been reduced to a tenth of that of the original solution. Length and breadth increase are promoted, and as a result when the water control gives a percentage volume increase of about 47 %, a tenth of the concentration of the standard solution gives one of about 92 %. The data also suggest that some stimu-

TABLE 1. EFFECTS OF UNTREATED AND HEATED STANDARD SOLUTION ON LENGTH (L), BREADTH (B), VOLUME (V), AND PERCENTAGE VOLUME INCREASE (PV)

IB indicates initial breadth, H water, E undiluted standard solution, and figures in first column the fractional concentration of this solution. NS indicates not significant, S significant at 5 % level, and HS at 1 % level.

	untreated (IB = 1.05 mm.)				heated (IB = 1.05 mm.)			
	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV
H	2.10	1.08	1.93	47.3	2.10	1.08	1.93	47.3
E	2.43	1.12	2.37	81.0	2.21	1.09	2.06	57.4
$1 \times 10^{-1}$	2.49	1.13	2.51	91.9	2.20	1.07	1.99	52.0
$1 \times 10^{-2}$	2.36	1.13	2.35	79.4	2.20	1.08	2.00	53.2
$1 \times 10^{-3}$	2.29	1.11	2.20	68.4	2.16	1.07	1.94	48.1
$1 \times 10^{-4}$	2.22	1.08	2.04	55.3	2.12	1.07	1.91	45.7
sig.	HS	S	—	—	NS	NS	—	—

lation to length, breadth and percentage volume increase may still be given when the standard solution has been diluted a thousandfold.

The effect of heating is shown by the data of table 1. It is evident that in contrast to the untreated solution the heated solution has little or no effect on length or breadth increase.

TABLE 2. EFFECT OF SAME STANDARD SOLUTION FRESH AND AFTER STORAGE FOR 24 HR

Symbols as in Table 1

	fresh (IB = 1.07 mm.)				after 24 hr. (IB = 1.05 mm.)			
	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV
H	2.17	1.09	2.02	50.2	2.10	1.08	1.94	49.3
E	2.26	1.10	2.17	61.3	2.23	1.10	2.12	63.1
$1 \times 10^{-1}$	2.41	1.14	2.47	83.7	2.22	1.11	2.16	66.0
$1 \times 10^{-2}$	2.23	1.12	2.19	63.4	2.17	1.08	2.00	53.8
$1 \times 10^{-3}$	2.21	1.13	2.23	65.9	2.09	1.08	1.91	47.3
$1 \times 10^{-4}$	2.17	1.10	2.08	54.6	2.09	1.07	1.87	44.0
sig.	HS	S	—	—	NS	NS	—	—

The effect of storage on the activity of the standard solution is shown by the data of table 2, which indicates that after 24 hr. storage the solution has little or no effect on length or breadth increase.

### Concentrate

The effects of freshly prepared solutions of the concentrate are shown by the data for the untreated solution in table 3 and by the first set of data in table 4. The data for the untreated solution of table 3 are reproduced in figure 2. The freshly prepared solution has a stimulating effect on length increase. The effect on breadth increase,

however, is only slight and is usually not statistically significant; nevertheless, it has been recorded repeatedly and is probably a characteristic feature of the action of the concentrate as it is of that of the standard. The percentage volume increase

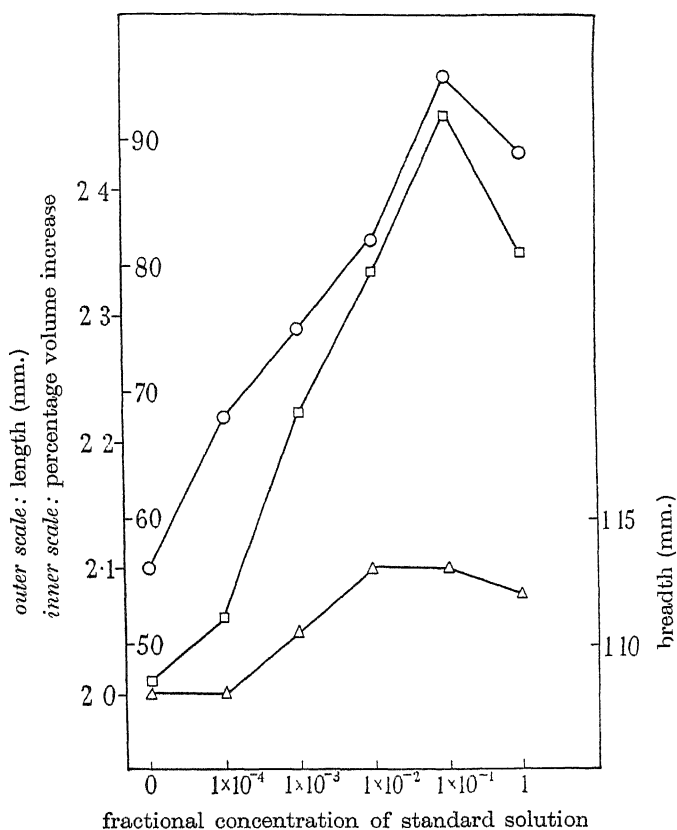


FIGURE 1. Effects with pea-root segments of various dilutions of standard solution on: ○ length, △ breadth, and □ percentage volume increase. Zero along abscissa indicates pure water.

TABLE 3. EFFECTS OF UNTREATED AND HEATED SOLUTIONS OF THE CONCENTRATE ON LENGTH (L), BREADTH (B), VOLUME (V) AND PERCENTAGE VOLUME INCREASE (PV)

IB indicates initial breadth in mm., H water, and figures in first column mg. of solute/l. NS indicates not significant, S significant at 5% level, and HS at 1% level.

	untreated (IB = 1.05 mm.)				heated (IB = 1.05 mm.)			
	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV
H	2.17	1.09	2.04	57.5	2.17	1.09	2.04	57.4
100	2.25	1.09	2.11	63.0	2.14	1.07	1.94	50.0
10	2.34	1.12	2.32	79.3	2.14	1.08	1.95	50.5
1	2.10	1.10	2.02	55.6	2.09	1.11	2.01	55.3
0.1	2.13	1.10	2.02	56.2	2.11	1.08	1.93	49.0
0.01	2.15	1.10	2.06	58.7	2.15	1.09	2.01	54.9
sig.	HS	NS	—	—	NS	NS	—	—

is greatest at concentrations of 10 mg./l., but is less than the greatest volume increase with the standard solution. This corresponds to the less pronounced effect on breadth increase, and may be taken to indicate that the activity of the solute has decreased during the process of extraction from the solution. It may be noted that

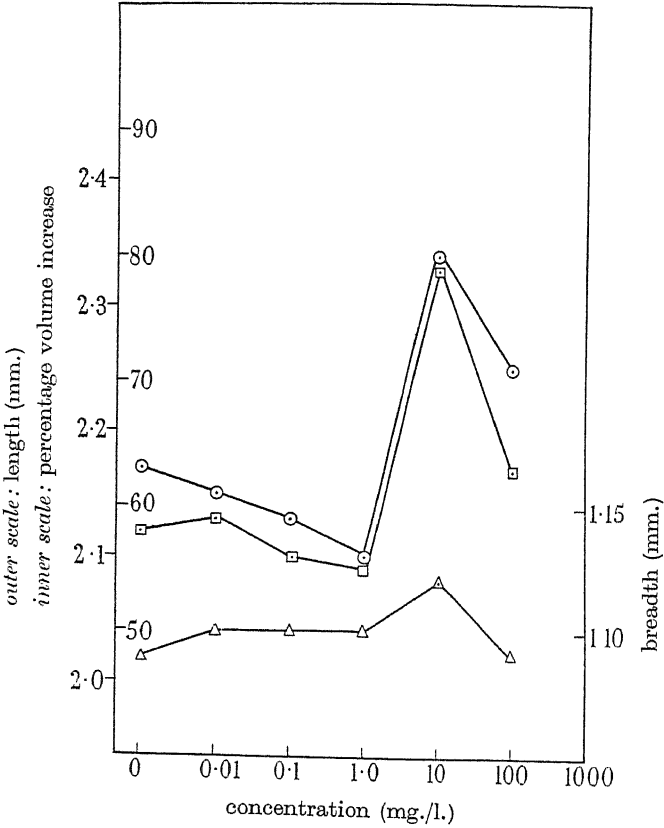


FIGURE 2. Effects with pea-root segments of various concentrations of the concentrate on: ○ length, △ breadth, and □ percentage volume increase. Zero along abscissa indicates pure water.

TABLE 4. EFFECT OF REFRIGERATOR STORAGE ON ACTIVITY OF CONCENTRATE SOLUTIONS

Figures 0, 1 and 2 at head of table indicate age of solution in days. Symbols as in table 3

	0 (IB = 1.10 mm.)				1 (IB = 1.10 mm.)				2 (IB = 1.06 mm.)			
	L	B	V	PV	L	B	V	PV	L	B	V	PV
	(mm.)	(mm.)	(mm. <sup>3</sup> )		(mm.)	(mm.)	(mm. <sup>3</sup> )		(mm.)	(mm.)	(mm. <sup>3</sup> )	
H	2.11	1.15	2.18	52.2	2.09	1.11	2.03	42.5	2.08	1.09	1.93	45.8
1000	2.26	1.18	2.47	72.5	2.16	1.14	2.21	55.5	2.18	1.12	2.15	62.4
100	2.26	1.19	2.52	75.8	2.19	1.17	2.36	65.5	2.18	1.12	2.16	63.7
10	2.32	1.18	2.52	76.3	2.15	1.14	2.18	52.8	2.09	1.09	1.94	47.0
1	2.18	1.16	2.28	59.5	2.15	1.13	2.15	51.2	2.06	1.10	1.96	48.0
0.1	2.11	1.15	2.19	53.2	2.09	1.10	2.00	40.4	2.07	1.10	1.96	48.6
0.01	2.13	1.14	2.19	52.7	2.07	1.11	2.00	40.0	2.11	1.10	2.00	51.8
sig.	HS	NS	—	—	S	S	—	—	NS	NS	—	—

the percentage volume increase is greater in the highest concentration of the preparation than it is in water, that it tends to increase with dilution until a peak value is reached, and then tends to decrease with further dilution.

The effect of heat treatment on the activity of the concentrate is shown by the data of table 3. While the untreated solution has considerable effects on length and breadth increase the heated solution has no effects whatever at any concentration.

The effect of refrigerator storage on the activity of the concentrate is shown by the data of table 4, which indicate that while the fresh solution enhanced the percentage volume increase from about 52 in water to about 76 with a solution containing 10 mg./l. of the concentrate, the solution when it was 24 hr. old had only a slight effect, and when it was 48 hr. old had little or none. Moreover, while the fresh solution had a considerable effect on length increase, the 24 and 48 hr. old solutions had little or no effect.

#### D-xyloketose

Three representative sets of results obtained with three freshly prepared solutions of the sugar are given in table 5. The data for solution 1 are reproduced graphically in figure 3, and a set of results obtained with maize-root segments is shown in figure 4. Other results with freshly prepared untreated solutions are given in tables 6 and 7.

TABLE 5. EFFECT OF FRESHLY PREPARED SOLUTIONS OF D-XYLOKETOSE

1, 2 and 3 at head of table indicate replicate experiments with solutions prepared from 3 different samples of sugar. Symbols as in table 3.

	1 (IB = 1.04 mm.)				2 (IB = 1.07 mm.)				3 (IB = 1.04 mm.)			
	L	B	V	PV	L	B	V	PV	L	B	V	PV
	(mm.)	(mm.)	(mm. <sup>3</sup> )		(mm.)	(mm.)	(mm. <sup>3</sup> )		(mm.)	(mm.)	(mm. <sup>3</sup> )	
H	2.09	1.08	1.91	50.0	2.15	1.10	2.04	51.6	2.33	1.08	2.12	65.7
1000	—	—	—	—	2.34	1.15	2.41	79.1	2.42	1.07	2.19	70.8
100	2.31	1.09	2.16	69.9	2.20	1.14	2.27	68.6	2.39	1.08	2.19	71.0
10	2.32	1.13	2.32	82.0	2.37	1.15	2.47	83.3	2.47	1.08	2.31	80.2
1	2.39	1.14	2.44	92.0	2.30	1.13	2.31	71.6	2.59	1.11	2.52	97.2
0.1	2.30	1.11	2.22	74.0	2.22	1.10	2.11	56.8	2.38	1.09	2.23	73.6
0.01	2.19	1.08	1.99	56.6	2.20	1.10	2.10	55.9	2.31	1.09	2.14	67.2
0.001	2.17	1.09	2.00	57.8	—	—	—	—	—	—	—	—
sig.	HS	HS	—	—	HS	HS	—	—	HS	NS	—	—

It is evident from the data of table 5 and figure 3 that D-xyloketose has a considerable stimulating effect on extension growth in low concentrations. The data show that the length and the breadth in the highest concentrations of the sugar are greater than they are in water, and that both tend to increase further as the concentration is reduced from 1000 to 10 or 1 mg./l. With further dilution, however, length and breadth tend to decrease. The same general trend is given by the data for percentage volume increase, which is greater with the highest concentration of the sugar than it is with water, increases with decreasing concentration until a peak value is reached with 10 or 1 mg./l., and then decreases progressively with

further dilution. It is significant, however, that with some samples considerable stimulation is given with 0.1 mg./l. which represents 1 part per 10,000,000 of water.

Figure 4 shows that stimulating effects are also given with maize-root segments, although there are apparently certain differences in the reactions of pea and maize segments to the sugar. In the highest concentrations of the sugar with maize, length and breadth are greater than they are in water, and breadth tends to increase

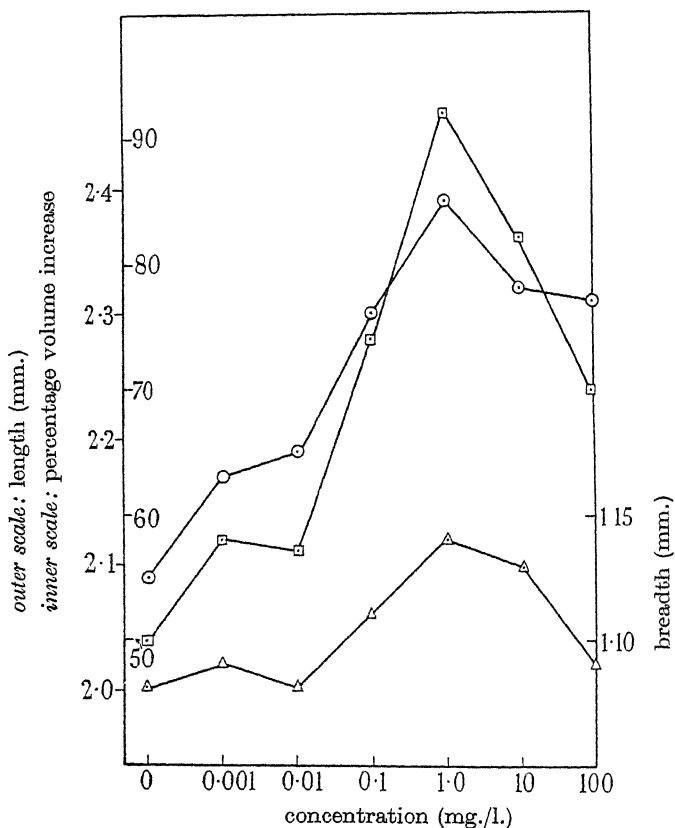


FIGURE 3. Effects with pea-root segments of various concentrations of D-xyloketose on: ○ length, △ breadth, and □ percentage volume increase. Zero along abscissa indicates pure water.

with dilution until a peak value is reached at a concentration of 10 mg./l. and then to decrease progressively with further dilution. On the other hand, length tends to decrease with dilution from the highest concentration. The resultant effects on percentage volume increase, however, give a position similar to that obtained with peas, in which the increase is enhanced with dilution until a peak value is reached and then decreases with further dilution. It may be noticed that with maize as with peas some stimulation is given at a concentration of 0.1 mg./l.

The effect of heating on the activity of xyloketose is shown by the data of table 6. It is evident that after heat-treatment the sugar has no stimulating effect whatever.

The effect of refrigerator storage is shown by the data of table 7, which indicates that although the activity is high on the first day, it is lower on the second, and still

lower on the third. The values for 48 hr. (2 days) are relatively high, but the control values (H) for this series indicate that this is due to an abnormally high capacity for extension in the segments used on that occasion. Moreover, whereas the length differences are significant on the first (0 days) and the second days (1 day) they are

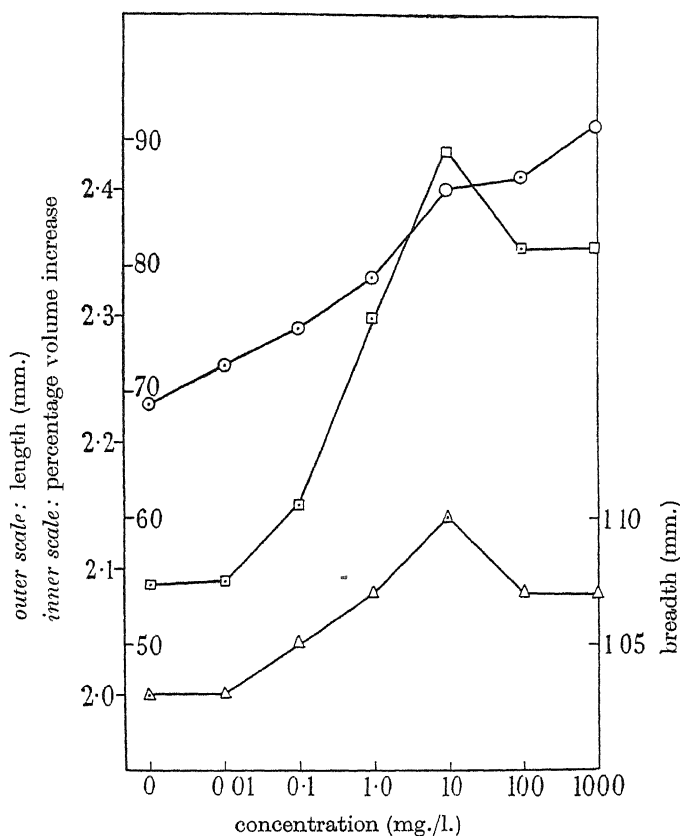


FIGURE 4. Effects with maize-root segments of various concentrations of D-xyloketose on: ○ length, △ breadth, and □ percentage volume increase. Zero along abscissa indicates pure water.

TABLE 6. EFFECT OF HEATING ON ACTIVITY OF XYLOKETOSE

Symbols as in table 3

	untreated (IB=1.07 mm.)				heated (IB=1.07 mm.)			
	L	B	V	PV	L	B	V	PV
	(mm.)	(mm.)	(mm. <sup>3</sup> )		(mm.)	(mm.)	(mm. <sup>3</sup> )	
H	2.12	1.12	2.09	54.9	2.12	1.12	2.09	54.8
1000	2.29	1.16	2.40	78.3	2.15	1.14	2.18	61.8
100	2.31	1.15	2.40	78.1	2.16	1.13	2.18	61.9
10	2.37	1.18	2.58	91.4	2.10	1.13	2.11	56.8
1	2.36	1.17	2.55	89.1	2.14	1.14	2.17	60.8
0.1	2.18	1.14	2.22	64.5	2.10	1.13	2.11	56.5
sig.	HS	NS	—	—	NS	NS	—	—

TABLE 7. EFFECT OF REFRIGERATOR STORAGE ON ACTIVITY OF D-XYLOKETOSE

Figures 0, 1 and 2 at head of table indicate age of solution in days. Symbols as in table 3

	0 (IB = 1.04 mm.)				1 (IB = 1.05 mm.)				2 (IB = 1.04 mm.)			
	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV	L (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV
H	2.12	1.08	1.93	50.1	2.24	1.08	2.06	59.3	2.47	1.08	2.26	78.4
1000	2.31	1.15	2.40	86.8	—	—	—	—	2.55	1.13	2.54	100.7
100	2.25	1.14	2.29	78.1	2.41	1.11	2.35	81.8	2.57	1.14	2.60	105.6
10	2.42	1.18	2.64	105.2	2.50	1.11	2.44	89.0	2.52	1.11	2.43	92.1
1	2.31	1.14	2.37	84.6	2.37	1.10	2.26	75.1	2.39	1.09	2.22	75.6
0.1	2.18	1.14	2.21	72.1	2.20	1.09	2.04	58.3	2.38	1.08	2.18	72.6
0.01	2.10	1.10	2.00	55.0	2.19	1.09	2.03	57.0	—	—	—	—
sig.	HS	HS	—	—	HS	NS	—	—	NS	NS	—	—

not so on the third. The control values for the fresh and for the 24 hr. old series are about the same, but whereas with the fresh solution the percentage volume increase at 10 mg./l. is 105 with the 24 hr. old solution it is 89.

It may be emphasized that it is improbable that the results given with freshly prepared solutions of D-xyloketose are due to impurities in the sugar samples used for the following reasons. The sugar has been prepared biologically from D-arabitol and chemically from D-xylose, and both methods of preparation have yielded samples which promote extension growth. Secondly, the effect of the sample of D-xylose from which the xyloketose was prepared has been examined, and it has been found that it has no growth-promoting properties whatever in low concentration. It was also found, however, that the xylose acts as a mass nutrient at high concentrations, since with solutions containing 1 g./l. extension is promoted. This indicates that the absence of an effect at low concentrations is not due to inhibitory effects of xylose.

#### DISCUSSION

It is clear from the data of tables 1 and 3 that both the standard solution and the concentrate contain some material which promotes the extension growth of pea roots. Moreover, it is evident that the material involved is the same in both preparations. With both, the segments increase in length and in breadth to a greater extent than they do with water, both are inactivated by heat, and the activity of both decreases rapidly with time. Clearly the active complex or substance is removed from the standard solution when this is treated with charcoal and is recovered from the eluate. At the same time the evidence indicates that the substance involved does not act as a mass nutrient. The highest activities with the concentrate are given with solutions containing 10 mg./l., which is a concentration of 1 part per 100,000 of water and is lower than that in which nutrient effects might be expected. The effect of diluting the standard solution is compatible with this interpretation, since it has been shown (table 1 and figure 1) that some activity is still given when the standard is diluted a thousandfold.

The similarities between the effects and the properties of D-xyloketose and those of the standard and the concentrate are close. D-xyloketose also stimulates



extension growth by promoting both lateral and longitudinal extension; solutions of this substance are also inactivated by heat, and they also lose their activity rapidly during refrigerator storage. These similarities in themselves suggest that the active component of the natural preparations is either the same as or similar to D-xyloketose. This conclusion, however, is supported by other evidence. The chemical examination of the concentrate in the investigation on the germination of *Striga* shows that it contains pentose sugars. Secondly, D-xyloketose gives greater stimulation than the concentrate, and at higher dilutions. Thus, while acting as a growth stimulator, D-xyloketose is more effective in this respect than the concentrate. Tables 3 and 5 and figures 2 and 3 show that with segments that give about a 50 % increase in volume in water an increase of only about 80 % is given with the concentrate and then only at a concentration of 10 mg./l. With concentrations of less than 10 mg./l. there is never any indication of any stimulation. D-xyloketose, on the other hand, with segments that again give about a 50 % increase in volume in water, gives increases in volume of about 100 % at concentrations of 1 to 10 mg./l. Moreover, some stimulation is frequently observed with solutions containing 0.1 mg./l.

Moreover, the conclusion that the substance in the standard solution and in the concentrate which stimulates extension growth is a sugar, is consistent with the results of certain observations on the effects of sugars other than D-xyloketose. It has been found that L-rhamnose and D-fructose also stimulate extension growth in low concentrations, and also promote both longitudinal and lateral growth. The effects of L-rhamnose are small and they are only given at a concentration of 100 mg./l.; those of fructose are larger and given at a concentration of 10 mg./l. The fructose effects, however, although greater than those of rhamnose, are smaller than those of xyloketose.

The properties of the standard solution of the concentrate and of D-xyloketose are strikingly similar in relation to the germination of *Striga* and to extension growth in root segments. In both connexions the solutions are active when fresh, are inactivated by heating, and they tend to lose their activity during storage. The effects of D-xyloketose indicate that the same substance in the natural preparations is involved in the two connexions and that this substance is acting on the same physiological process in the two tissues. Differences in the reactions of the two systems have, however, been noted which may be of some significance. The *Striga* seed reacts not only to D-xyloketose, D-fructose and L-rhamnose, but also to L-sorbose. The root segments, on the other hand, are unaffected by L-sorbose at any concentration. A further difference has been noted in the reactions to D-xyloketose. In the investigation on the influence of this sugar on the germination of *Striga* it was found that the results given with different samples regenerated from the same crystalline sugar derivative may differ considerably. A similar variation has been noted in connexion with the root system, but the variation is strikingly smaller. Some samples that have given no results with the parasite seed have given large stimulating effects with the root segments, and the number of samples that have given no effect with the segments is a negligible proportion of the total number tested.

It is evident that in the experimental situation analyzed here, a stimulator, which may be a sugar or derivative of a sugar, is released from a root and promotes extension growth in the tissues of other roots. The significance of this observation for the elucidation of the general process of root growth is difficult to assess from the present series of data. It is, however, of some importance in this connexion that it has been shown that the stimulator is produced by a large variety of roots and that in low concentration it promotes extension in the roots of very dissimilar species such as peas and maize (although it has been tested with segments of *Cucurbita Pepo* and has given no results with these). These observations suggest that the substance is of wide occurrence and that it stimulates extension growth in a variety of roots. Moreover, it has been found that D-xyloketose gives greater percentage volume increases although at higher concentrations with pea root segments than does heteroauxin. The effect of heteroauxin has been examined with the normal technique and a typical series of results are shown in table 8. At a concentration of 0.01 mg./l. heteroauxin gives a percentage volume increase of about 77 %, whereas D-xyloketose may give one of 100 % at 1.0 mg./l. It may be noted that the range of concentration over which D-xyloketose gives the greatest stimulation with root segments is about the same as that over which heteroauxin gives maximum stimulating effects with coleoptile segments. Thus it may be that the stimulating effects of sugars in low concentrations are at least as important as those of auxin in the extension growth of the root.

In this connexion it is of some interest that the effect of heteroauxin is qualitatively different from that of D-xyloketose. With heteroauxin stimulation involves only the longitudinal component. The breadths of segments treated with this hormone are all less than those of the water controls. This effect has been noted in all our experiments with heteroauxin.

TABLE 8. EFFECTS OF HETEROAUXIN ON GROWTH OF SEGMENTS

Symbols as in table 3 (IB=1.05 mm.)

	P (mm.)	B (mm.)	V (mm. <sup>3</sup> )	PV
H	2.23	1.10	2.12	62.9
10	1.87	1.06	1.66	28.2
1	1.98	1.08	1.82	39.9
0.1	2.18	1.08	1.88	52.8
0.01	2.46	1.09	2.30	77.1
0.001	2.45	1.08	2.23	71.7
0.0001	2.40	1.07	2.17	67.4
0.00001	2.23	1.09	2.06	58.7

The natural stimulant in this investigation has been obtained from seedlings of *Sorghum*, but it has also been obtained from intact growing roots of peas and maize, segments of which have been shown to react to the substance. The experimental observations suggest that the stimulator, while being utilized by cells in the extending zone, may be synthesized in some other region of the root, and that the amount that escapes from the root represents a portion that diffuses out of it in the course

of the flow to the zone of utilization. This interpretation depends on the assumption that the extending cells do not synthesize the appropriate carbohydrates as they apparently do not in the conditions of the present series of experiments. The evidence at present available, however, does not exclude the alternative possibility that such substances may be formed in extending cells which are provided with high nutrient levels of carbohydrate.

Finally, we wish to emphasize that while the present series of experiments show that one particular sugar, D-xyloketose stimulates extension growth in low concentrations, and that a substance which gives similar effects and is a sugar or a closely allied substance is released from roots, the experiments do not show that in natural root exudates one sugar alone is involved or that the sugar is even identical with D-xyloketose.

We wish to acknowledge our indebtedness to the Colonial Products Research Council for financial support, and to Mr A. G. Long for certain of the D-xyloketose preparations used in this investigation.

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# Crystalline anti-pernicious anaemia factor from liver

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[PLATE 27]

A red crystalline substance, intensely active against pernicious anaemia, has been isolated in very small yield from ox liver. It is probably identical with vitamin B<sub>12</sub> isolated a few weeks previously in America. The procedures employed were almost all physical rather than chemical. They included repeated adsorption on charcoal and elution with hot 65 % alcohol, adsorption chromatography on alumina, silica and charcoal, and partition chromatography between butanol or other solvents and moist silica; proteolysis to break down peptide impurities, fractional salting-out with ammonium sulphate and precipitation with phosphotungstic acid were generally also necessary before the substance could be crystallized from aqueous acetone.

A second red factor, also active both clinically and microbiologically, has been separated by partition chromatography on starch or filter paper, but has not yet been crystallized.

A number of physical measurements have been made. These include crystallography and X-ray crystallography, measurement of refractive indices, absorption spectrum, optical rotation, electrometric titration, electrical conductivity and mobility, and polarography.

Chemical analyses have been made for the elements C, H, N, P and Co. The presence of 4.0 % of cobalt leads to a molecular weight of 1500 for the dried substance, in agreement with the values from X-ray crystallography.

The anti-pernicious anaemia factor is slowly destroyed by cold dilute acid or alkali, by light and by strong oxidizing or reducing agents.

Attempts to induce chemical exchange with radioactive phosphate or radioactive cobaltous chloride, or with a radioactive cobaltamine, were unsuccessful.

An extensive literature review on the purification of the anti-pernicious anaemia factor of liver was published by Subbarow, Hastings & Elkin in 1945. For this and other reasons, it is only necessary to refer briefly to previous work. The historic discovery of the efficacy of oral liver in pernicious anaemia was made by Minot & Murphy in 1926. The early work of Cohn, West, Castle and others led to the preparation of liver extracts suitable for oral use, and later, after further purification, for injection. Dakin and others were able to effect still further purification. More active preparations were obtained by Subbarow, Jacobson & Prochownik (1936), by Laland & Klem (1936) and by Karrer, Frei & Ringier (1938). Subsequent to Subbarow's review, Emery & Parker (1946) reported on a preparation fully active clinically in a single dose of only 1 mg. Much chemical work on such fractions has been reported; lack of agreement between the results of the various groups no longer appears surprising, since it is now clear that none of these preparations contained more than 1 % of the active principle. This disappointing outcome of nearly twenty years' research is doubtless due to the exceptional difficulties of the problem. The active principle is so remarkably potent that fresh ox liver contains only about one part per million. Further, it seems devoid of characteristic chemical

properties that would assist in its separation, so that mainly physical methods have to be used. In our hands the key technique has been chromatography, and in particular partition chromatography (Martin & Synge 1941).

Much more effective in retarding progress than these chemical difficulties, however, have been those of assay. Several assay techniques involving animals have been described, but each in turn has proved unsatisfactory.

Until very recently the only reliable means for assessing the activity of preparations has been the clinical test on human pernicious anaemia patients in relapse. The paucity of suitable cases, the time taken by each test and the inevitable variations between individual responses have been among the factors hampering progress. In the present work we have been exceptionally fortunate in having the collaboration of Dr C. C. Ungley, of the Royal Victoria Infirmary, Newcastle-on-Tyne, who has carried out for us over a hundred painstaking clinical tests on experimental fractions.

Too recently to be of any assistance to us, a microbiological assay method has been mentioned in American publications (Shorb 1948; Shorb & Briggs 1948). It was this assay method that enabled a research team at Merck and Co. Inc., Rahway, New Jersey, to make rapid progress towards isolation of a red crystalline factor, which they have called 'vitamin B<sub>12</sub>' (Rickes, Brink, Koniuszy, Wood & Folkers 1948a; West 1948).

Early in 1947 we had obtained a number of amorphous pink preparations, which appeared to be chromatographically homogeneous and gave full clinical responses with doses around 0.5 mg. At the time we believed these preparations to be approaching purity, but a little before the American announcement we had obtained evidence that impurities present could be broken down by proteolysis, permitting extensive further purification (Smith 1948a). A few weeks later (Smith & Parker 1948) we obtained crystalline material probably identical with that obtained by Rickes *et al.*

#### FRACTIONATION METHODS

For the earlier stages of fractionation we followed methods developed by our colleagues Hurran & Emery (Emery & Parker 1946). These methods in turn were based on some devised by the Norwegian workers Laland & Klem (1936 and personal communications). Modifications were introduced from batch to batch, but a typical procedure is indicated in figure 1. The essential steps comprise extraction of the minced liver with aqueous alcohol, adsorption of the concentrated extract on activated charcoal, followed by thorough washing to remove less strongly adsorbed impurities, and elution of the charcoal adsorbate with hot 65% ethyl alcohol. The adsorption, washing and elution were repeated with less charcoal and eluant. Liver extract manufactured from ox liver proteolyzed with papain was used for later batches; it was introduced into the fractionation scheme at the point indicated in figure 1.

#### *Partition chromatography*

The most valuable new procedure that can be performed directly upon concentrates prepared by the scheme shown in figure 1 is partition chromatography.

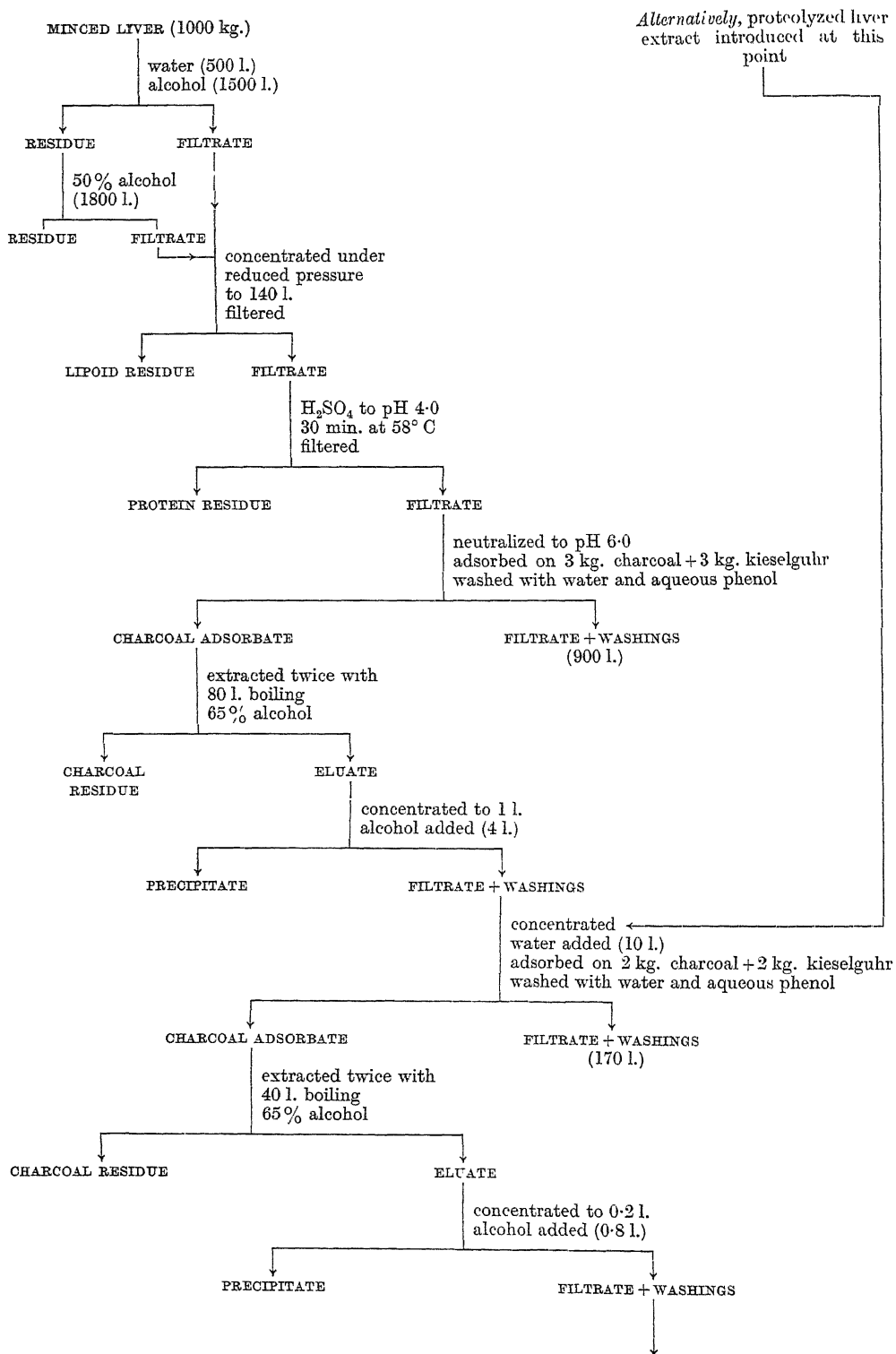


FIGURE 1

From what was known of the solubilities of the anti-pernicious anaemia factor, it seemed likely that partition chromatography with the solvent pair butanol and water would prove satisfactory. Our first attempts to prepare chromatographic columns with damp silica and *n*-butanol saturated with water were, however, frustrated because the columns became sticky and impervious. The water in the system is rapidly redistributed between the silica and the solvent so that water-saturated butanol makes the silica too wet; butanol containing 11 to 12 % of water, i.e. slightly more than half-saturated, is satisfactory for development. The simplest way to introduce sufficient water into the silica initially was to stir it with ten times its weight of *n*-butanol saturated with water and to use this mixture directly for making up the column.

The liver concentrates were applied to the column in one of two ways: (1) a very strong aqueous solution of the concentrate was extracted exhaustively with dry butanol, and the extracts were poured on to the column; or (2) the liver concentrate dissolved in the maximum volume of water was mixed with about three times its weight of dry silica, and the resulting powder was applied to the column after suspension in a little butanol.

Partition chromatograms made in this way and developed with butanol containing about 11 % of water quickly showed three coloured zones. A fast-moving yellow zone could easily be washed out completely from the column on continued development. This was followed by a pink zone which, after travelling a short distance down the column, moved very slowly on continued development. Finally, a brown zone remained at the top of the column and hardly moved at all on development. This behaviour made it clear that the silica was not functioning merely as an inert support for the water phase in the partition chromatogram; adsorption on the silica also played a part in retarding the development of the pink and brown zones. A series of clinical trials showed that nearly all the anti-pernicious anaemia activity was concentrated into the pink zone (from which it could be eluted with 50 % ethanol); when the zones above and below the pink one were tested, they were usually found to have only about 5 % of the total activity. Eventually we were able to draw the conclusion that the red colour was inseparable from the clinical activity by all the methods of fractionation we employed. From this point the later stages of purification were greatly simplified because it became possible to use colorimetry as a rough method of assay.

A number of solvents were tried in place of butanol for partition chromatography. Mixtures of phenol or cresol with butanol or other solvents were satisfactory, but little superior to butanol alone, except that development was somewhat more rapid. A mixture of butanol (containing 11.5 % of water) and phenol (liquefied with 8 % of water), in the ratio 3:1, produced an almost transparent column, doubtless because the refractive index of the silica approximated that of the solvent mixture. The only other solvents that proved specially advantageous were *n*- or *iso*-propanol containing 10 to 25 % of water. Although these alcohols are completely miscible with water, they appear capable of forming systems of two liquid phases with water absorbed in silica gel, since the chromatographic band system that developed closely resembled that obtained with butanol. Propanol containing about 50 % of

water eluted all the zones from the column indiscriminately. Our usual practice was to combine the main pink fraction from a chromatogram with that recovered by reworking the top brown fraction, and then to purify further by repeating the chromatography with a smaller column and often a different solvent.

### *Adsorption chromatography*

The observation that precipitated silica was capable of adsorbing the active principle prompted us to try adsorption chromatography from aqueous solutions. Fairly good results were obtained with the more strongly adsorptive batches of silica. A clearly defined, though faint, pink zone could be dissected from the extruded column after long development.

Tiselius (1948) has shown that in chromatography from aqueous solutions it is sometimes advantageous to use, instead of water, solutions of salts such as ammonium sulphate, insufficiently concentrated to salt-out the substances to be separated. We found that ammonium sulphate solutions up to about half-saturated could be used for the chromatography of liver fractions on silica. As the ammonium sulphate concentration was increased, the pink band became more sharply defined and travelled more slowly. It then becomes possible to decrease materially the size of column required for a given amount of liver concentrate. This was, however, the only advantage gained. The degree of purification effected was no better than when using water as solvent.

Purification by adsorption on charcoal, a procedure originated by Laland & Klem (1936) was indispensable in the preliminary purification of crude liver extract, and this step was usually repeated. At a much later stage of the purification we again found adsorption on charcoal to be advantageous. The whole of the activity could then be adsorbed from aqueous solutions on to an amount of charcoal only five or ten times the weight of the liver fraction. This operation was advantageously carried out chromatographically using a 5 to 10 mm. bed of charcoal (mixed with a little kieselguhr to expedite filtration) on a small filter. Some purification from less strongly adsorbed impurities could be effected by thoroughly washing the bed with water and then with water containing 10 % of propanol. Following this, the red active principle could be eluted by passing through the bed a comparatively small volume of a 1:1 mixture of water and *n*- or *iso*-propanol. The presence of more strongly adsorbed brown impurities could be demonstrated by finally eluting the charcoal bed with phenol.

At a late stage of this investigation we became aware of British Patent 590,956 (Boots Pure Drug Co., Short & King 1947). It claims that liver extracts can be purified by passing their aqueous solutions through a column of alumina. The alumina is stated to retain impurities, while the active principle passes through. We have confirmed the value of this technique and have sometimes used it with advantage before partition chromatography. With liver extracts of sufficient purity, the first runnings from an alumina column are pink in colour, but the pink zone is quickly overlapped by a yellow one.



*Non-chromatographic fractionation methods*

There remain to describe a number of other methods of purification not involving chromatography. We found in 1946 that very little of the red active principle was extracted from neutral aqueous solution by butanol, even after saturation with sodium chloride, though it was extracted after saturation of the aqueous layer with ammonium sulphate, and acidification to about pH 4. It appeared that these observations could be made the basis of a fractionation procedure. A liver concentrate was saturated with sodium chloride, and impurities were removed by extracting several times with butanol, these extracts being washed in turn with a very small volume of brine. The aqueous residue and brine washings were then saturated with ammonium sulphate, acidified to pH 4 and again extracted several times with butanol, leaving other impurities in the aqueous residue. The activity was recovered from these butanol extracts by washing with water containing sufficient sodium hydroxide to bring the pH to 7 or 8. This procedure, applied to a liver concentrate fully active in a dose of 16 mg., proved highly effective. About 80 % of the activity was recovered and the purity increased about fourteen-fold. The side fractions were also assayed clinically, and each was found to contain barely 5 % of the original activity. The value of extraction with butanol in presence of ammonium sulphate appears to have been rediscovered by Ellis, Petrow & Snook (1949).

It is often found in the purification of natural substances that their solubilities are markedly affected by the presence of impurities. This phenomenon has been encountered several times in the course of purification of the anti-pernicious anaemia factor and has led to much waste of effort. For example, the fractionation procedure with butanol and salts, though so effective with adequately purified starting material, failed almost completely with a somewhat cruder liver extract containing about four times as much solids per dose. Another example concerns solubility in alcohol. Earlier workers in the field have consistently used precipitation with alcohol to 90 or 95 % concentration as a means of separation from more soluble impurities. Yet the crystalline factor, and even preparations containing no more than 10 % of it, are readily soluble in 99 % alcohol. Again, as mentioned above, charcoal adsorbates from adequately purified liver concentrates can be completely eluted with 50 % propanol; but this eluting agent is almost useless when applied to charcoal adsorbates from relatively crude liver extracts.

Several of the early workers in this field precipitated impurities with lead acetate or basic lead acetate. We have found these reagents of some value, although in agreement with Dakin, Ungley & West (1936) we find that basic lead acetate tends to bring down part of the active principle.

Dakin & West (1935) found that activity could be precipitated from crude liver extracts by saturation with ammonium sulphate at neutral or faintly acid reaction. We find that this property of insolubility in nearly saturated ammonium sulphate persists throughout the course of fractionation. At a suitable late stage a trace of impurity is sometimes thrown out at one-third saturation, while the active principle is almost completely precipitated at two-thirds saturation, leaving other impurities in solution.

Laland & Klem (1936) reported little success in attempts to fractionate purified liver concentrates in ethyl or methyl alcohol by addition of ether. We have found, on the contrary, that with more highly purified preparations a fraction of low solubility in alcohol can sometimes be separated by dissolving in warm alcohol, cooling and filtering; the active principle can then be precipitated almost completely by adding an equal volume of ether, leaving other impurities in solution.

Cohn, Minot, Alles & Salter (1928) and also West & Nichols (1928) found that activity could be precipitated with phosphotungstic acid. We have confirmed the utility of this reagent at a much later stage in the purification. The red phosphotungstate was completely soluble in acetone, sometimes leaving a small insoluble brown residue. The phosphotungstate was decomposed by shaking a suspension in aqueous acid with a mixture of amyl alcohol and ether. The active principle was conveniently separated from the aqueous acid by extraction into phenol, from which it could be recovered in turn by diluting the phenol with about 5 volumes of ether and extracting with water.

Combinations of most of these techniques were applied to various lots of liver concentrate, giving amorphous pink material with a fairly consistent level of clinical activity, namely, 0.3 to 0.6 mg. as the minimum amount necessary for an optimal response in a case of pernicious anaemia. Losses of activity occurred, as is usual in the purification of labile naturally occurring substances, but in favourable instances the yield from proteolyzed liver extract, in terms of clinical activity, was roughly 25 %.

Repeated application of partition or adsorption chromatography on silica failed materially to increase the purity, and at one time we were inclined to accept this as circumstantial evidence for homogeneity. Use of the Tiselius electrophoresis apparatus demonstrated, however, that such a preparation was in fact far from homogeneous. We then found that digestion with mixed bacteria, or more conveniently with trypsin or duodenal enzymes or both, effected degradation of persisting peptide impurities and that separation from the resulting amino-acids or simpler peptides then became possible. The most useful techniques following proteolysis were partition chromatography, adsorption chromatography on charcoal, fractional precipitation with ammonium sulphate and precipitation with phosphotungstic acid.

### *Crystallization*

After adequate purification by these means, it finally proved possible to crystallize the active substance from aqueous acetone (see figure 2). Reasonable yields of crystalline material have been obtained (following seeding) from concentrates containing only about 10 % of the active principle. Pure crystalline material was best obtained by treating a concentrated aqueous solution with about 2 volumes of acetone, removing a trace of flocculent material by centrifuging and then slowly adding acetone to about 85 % concentration. On standing, a precipitate of crystals, sometimes mixed with red gum, appeared. Several recrystallizations could be carried out with little loss by dissolving in the minimum of water and again adding acetone to about 85 % concentration. Further losses were incurred in these later

steps, and the yield of the pure crystalline factor seldom exceeded 15 mg./1000 kg. of fresh liver.

It is a pleasure to record our indebtedness to Mr N. C. Jeffery and Mr R. W. Peevers for the processing of many large batches of liver.



FIGURE 2. Crystals of anti-pernicious anaemia factor in mother-liquor ( $4/3 \times$  actual size).

#### EVIDENCE FOR A SECOND RED FACTOR

Partition chromatograms on silica occasionally showed signs of a second small pink zone. This was revealed much more unequivocally on partition chromatograms with air-dried starch as the base and *n*-butanol 9/10 saturated with water as mobile phase. From an early batch of concentrate made from non-proteolyzed liver, the main pink zone travelled with an *R* value (see Martin & Synge 1941) of about 0.5, while the minor pink zone travelled much faster with an *R* value of about 1.2. Both zones were rather diffuse. The two zones travelled at about the same rates on a column of starch impregnated with a phosphate buffer at pH 3. The two pink fractions travelled at their characteristic rates when applied separately to two micro-columns. Thus the two red substances appear to be distinct, though they may well be closely related chemically. Both fractions were clinically tested and both were found active.

Another preparation from proteolyzed liver also gave two zones travelling at about the same rates, but now the major zone was the fast one and the minor zone the slow one. It is not yet clear whether this reversal of proportions results from the proteolytic step or from differences in the original liver.

This separation could also be readily effected by partition chromatography on filter paper with water-saturated *n*-butanol as mobile phase, and ascending or descending development. The  $R_F$  values (see Consden, Gordon & Martin 1944) were

very small. The crystalline factor after several recrystallizations showed only a single spot, but less pure preparations and mother-liquors from the crystallization showed a second smaller red spot. Clearly visible pink zones on paper, or on silica or starch columns about 3 mm. in diameter, were produced by 10  $\mu$ g. amounts of these pigments. In collaboration with Cuthbertson & Smith (1949) we have shown that very much smaller quantities can equally well be separated on paper, the spots being detected by application of the developed strip to a layer of suitable nutrient agar seeded with *Lactobacillus lactis* Dorner; on incubation, microbiologically active factors gave rise to circles or ellipses of growth. In this way it was shown that both red pigments are microbiologically active. In addition, liver extracts gave rise to two faster-moving components having microbiological activity differing in degree from that shown by the two red components; whereas the latter produce zones of dense growth on the agar plates, these fast components give only thin diffuse growth. One of these substances remains unidentified, but the faster-moving one is thymidine, which was already known to simulate vitamin B<sub>12</sub> in the microbiological assay (Shive, Ravel & Eakin 1948). Thymidine is clinically inactive (Ungley 1949a).

We have found recently that secondary butanol is much superior to *n*-butanol for this paper-strip chromatography. The  $R_F$  values are greater and the red zones separate well. With this solvent we have been able to resolve the slow-moving red factor into two microbiologically active components. In addition, we have found two microbiologically inactive, but red components in mother-liquors from the crystallization of the anti-pernicious anaemia factor; these are presumably degradation products. Finally, the red product arising from hydrolysis of the anti-pernicious anaemia factor with hot acid (see later) travels almost at the solvent front in these paper-strip chromatograms. These factors are listed in table 1 with what is known of their properties.

TABLE 1. FACTORS SEPARATED BY CHROMATOGRAPHY ON PAPER  
WITH SECONDARY BUTANOL

rate of travel	name	colour	microbiological test	clinical trial
slow				
↓	'slow red component' {	red	active	} active
		—	active	
		red	inactive	
	crystalline anti-pernicious anaemia factor	red	active	active
	degradation product (?)	red	inactive	not tested
	riboflavin	yellow	inactive	inactive
	unidentified substance	colourless	slightly active	not tested
	thymidine	colourless	slightly active	inactive
fast	hydrolysis product	red	not tested	not tested

#### PHYSICAL AND CHEMICAL PROPERTIES OF THE CRYSTALLINE ANTI-PERNICIOUS ANAEMIA FACTOR

The factor crystallizes from aqueous acetone or from extremely concentrated aqueous solutions as dark red needles. Crystals several millimetres in length have been obtained.

*Crystallography and X-ray crystallography*

The investigations made at the University of Oxford by Dr Dorothy Hodgkin, Dr M. W. Porter and Mr R. C. Spiller are described in an appendix to this paper.

The X-ray crystallography is of particular interest in providing a figure for the molecular weight. Dr Hodgkin's value is about 1600 for the air-dried substance.

Mr Spiller has found that the refractive indices agree well with those recorded for vitamin B<sub>12</sub> by Rickes *et al.*\*

*Absorption spectrum*

The absorption spectrum of the crystalline material is shown in figure 3. The ultra-violet portion of the curve was determined with the Hilger medium spectrograph. The visible portion was very kindly determined by Mr R. Donaldson of the National Physical Laboratory, using a General Electric recording spectrophotometer. Almost

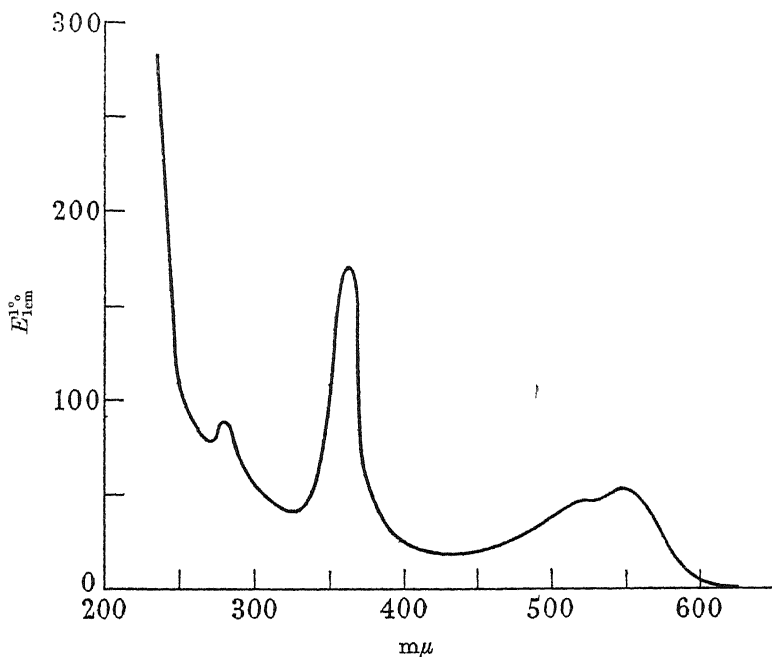


FIGURE 3. Absorption spectrum (ultra-violet and visible) of crystalline anti-pernicious anaemia factor in aqueous solution.

\* [Note added in proof.] Since submitting this paper we have been able through the courtesy of Dr R. Major of Messrs Merck and Co. Inc., to exchange specimens of our crystalline anti-pernicious anaemia factor and their vitamin B<sub>12</sub>. The two specimens are identical in all the following respects: cobalt and phosphorus content; ultra-violet and visible absorption spectra; single crystal X-ray diagrams; refractive indices; polarographic step; behaviour on paper chromatograms; microbiological and clinical activities.

Discrepancies between our analytical figures and molecular weight values, and those recently published by Brink, Wolf, Kaczka, Rickes, Koniuszy, Wood & Folkers (*J. Amer. Chem. Soc.* 1949, 71, 1854), can be explained as follows. It appears that our cautious drying at 56° C *in vacuo* did not completely remove water of crystallization (as we have confirmed with subsequent batches); the analyses agree well if it is assumed that our material as analyzed retained 6 or 7 molecules of H<sub>2</sub>O.

identical results were obtained with a photoelectric spectrophotometer kindly lent to us by Messrs Hilger and Watts. All the measurements were made in neutral aqueous solution, but an acid solution (pH 1.7) and an alkaline solution (pH 11.7) gave identical readings in the visible and almost identical ones in the ultra-violet part of the spectrum. This curve was demonstrated at a meeting of the Biochemical Society in July 1948. That published by Ellis *et al.* (1949) is in excellent agreement with ours.

#### *Infra-red absorption*

The infra-red absorption spectrum of a single minute crystal of the factor has been determined (Barer, Cole & Thompson 1949) with the Burch reflecting microscope harnessed to an infra-red spectrophotometer. The absorption curve is reproduced in figure 4.

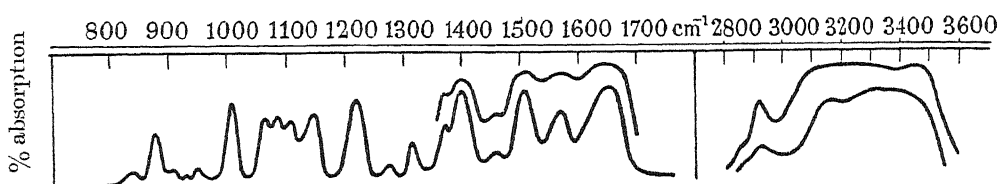


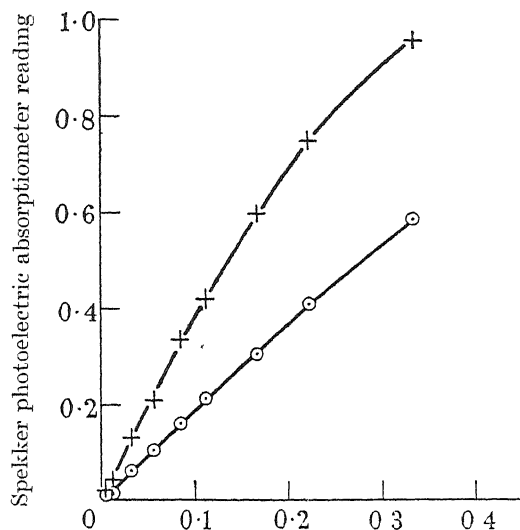
FIGURE 4. Infra-red absorption spectrum of crystalline anti-pernicious anaemia factor.

#### *Colorimetric determination*

Colorimetric estimations of the anti-pernicious anaemia factor in pure or nearly pure preparations were conveniently made with the Hilger photoelectric absorptiometer, using the micro-cell holding 0.8 ml. The Ilford green (no. 604) gelatine filter (maximum absorption at  $520\text{ m}\mu$ ), protected by a heat-resisting filter (H 503), was found most suitable. The calibration curve is shown in figure 5. Another calibration curve was constructed with the Chance blue glass filter (O.B. 1) which has maximum transmission at  $440\text{ m}\mu$  corresponding approximately with a trough in the absorption curve of the anti-pernicious anaemia factor. Colorimetric estimations were unreliable if the ratio of the values with the two filters fell much below 1 (due to the presence of yellow impurities). Since the factor is rather easily inactivated with only partial loss of red colour, colorimetric estimations can be misleading.

#### *Optical rotation*

Determination of the optical rotation of the anti-pernicious anaemia factor presented considerable difficulty on account of the intense colour of its solutions. It was not possible to get sufficient light through a solution of suitable concentration using a laboratory-type mercury, sodium or cadmium lamp. We were finally able to obtain readings with an experimental high-intensity cadmium lamp kindly lent to us by Mr B. S. Cooper of the Research Laboratories of the General Electric Co. For a solution of 1.98 mg. in 0.4 ml. of water in a 5 cm. micropolarimeter tube, the specific rotation at  $20^\circ\text{C}$  at  $643.8\text{ m}\mu$  was  $-110^\circ \pm 10\%$ . This substance is undoubtedly laevo-rotatory, but it does not have the exceptionally high optical rotation shown by some co-ordination compounds of cobalt.



concentration of anti-pernicious anaemia factor in mg./ml.

FIGURE 5. Calibration curve for the determination of the anti-pernicious anaemia factor.

x, Ilford filter, no. 604; o, Chance filter, no. O.B. 1.

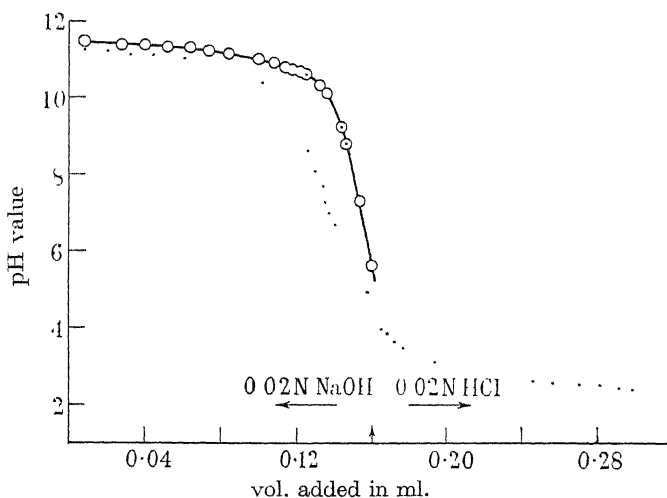


FIGURE 6. Electrometric titration of anti-pernicious anaemia factor. 3.89 mg. of crystalline anti-pernicious anaemia factor were dissolved in 0.5 ml. of distilled water and titrated electrometrically to pH 11.45 with 0.02N-sodium hydroxide (from a Conway microburette), and then back-titrated to pH 2.40 with 0.02N-hydrochloric acid. The titration was conducted at 20° C, and a glass-calomel electrode system connected to a Cambridge pH meter was used for the pH measurements.

#### *Electrometric titration*

Electrometric titration (figure 6) shows that the anti-pernicious anaemia factor does not contain any strongly acidic or basic groups. The acid back-titration curve does not superimpose completely on the original curve; this may indicate that some decomposition has taken place in the strongly alkaline solution, or it may be due merely to absorption of atmospheric carbon dioxide.

*Electrical conductivity*

The anti-pernicious anaemia factor appears to be weakly ionized in aqueous solution. At a molar concentration of  $2.2 \times 10^{-4}$  the specific conductivity at  $25^\circ \text{C}$  was 11.8 gemmhos. This corresponds to a molar conductivity of only 53, assuming a single ionizing group, or a proportionately lower figure if, as is probable, there is more than one ionizing group in the molecule.

*Electrical mobility*

An ionophoresis experiment was carried out in the apparatus described by Consden *et al.* (1946). A trough of silica gel was prepared in acetate buffer at pH 7.0 and stiffened with paper pulp. When the gel had set, a transverse trough approximately 1 cm. in width was cut out and refilled with another portion of similar silica gel containing about 1 mg. of the anti-pernicious anaemia factor in an incompletely purified condition. On application of a direct-current potential of 300 V, a red zone very slowly migrated from the trough towards the cathode, leaving yellow impurities in their original position. Allowing for endosmosis the net movement is towards the anode with a mobility of approximately  $1.7 \times 10^{-5} \text{ cm.}^2/\text{V}/\text{sec.}$

*Polarography*

The crystalline anti-pernicious anaemia factor was first examined polarographically by Dr E. Kodicek to whom our thanks are due; we have since confirmed and extended his findings.

In neutral solution there is a well-defined polarographic step with a half-wave potential of  $-1.53 \text{ V}$  (against the saturated calomel electrode). The step is surmounted by a characteristic maximum (figure 7). The position and height of the

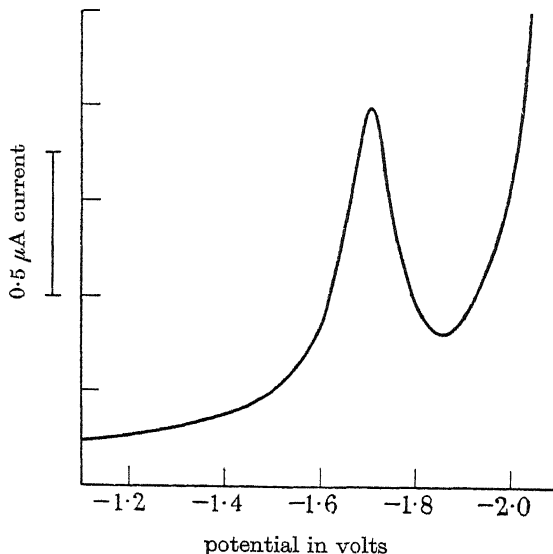


FIGURE 7. Polarogram for 0.025M-phosphate buffer solution containing 0.012 mg. of the anti-pernicious anaemia factor per ml. At a pressure of 48 cm. of mercury and at  $25^\circ \text{C}$ , the drop-time ( $t$ ) of the dropping mercury electrode on open circuit in 0.1N-potassium chloride was 3.50 sec., the weight of mercury dropping per second ( $m$ ) was 1.60 mg., and  $m^{3/2}t^{1/2}$ , 1.69.



step varies with pH value, the half-wave potential becoming more negative and the height much lower in alkaline solutions. For solutions in a 0.025 M-phosphate buffer at pH 7.4, there is an approximately linear relationship between concentration and the height of the maximum over the concentration range, 0.005 to 0.020 mg./ml. Polarography is not recommended for the estimation of vitamin B<sub>12</sub>, since a small amount of a surface-active substance would seriously affect the height of the maximum.

### *Molecular weight*

Several attempts were made to get an approximate measure of the molecular weight before crystalline material became available. Although the ultra-centrifuge was thought unlikely to give a quantitative result with a molecule of the dimension anticipated, Dr R. A. Kekwick and Dr B. R. Record kindly agreed to carry out a determination at the Lister Institute. The impure material then available gave no sedimentation boundary but only a concentration gradient even at 250,000 g; this suggests a molecular weight probably well below 10,000.

Three experiments were carried out to determine the approximate molecular weight by the diffusion method (Northrop & Anson 1929). This method, though of low accuracy, is useful because it should be unaffected by the presence of other substances, provided the analytical method employed is specific for the substance under examination. We therefore felt justified in applying the technique to partially purified material (now known to be about 3 % pure), estimating the anti-pernicious anaemia factor colorimetrically. The value obtained was approximately 3000. The determination was repeated with more highly purified material proteolyzed with both papain and trypsin; we had reason to believe this treatment might have split off some amino-acids from the original substance. The value obtained, however, was again approximately 3000.

This diffusion method was not applied to the pure crystalline material when it became available, because other and more accurate methods of molecular weight determination could be employed.

### *Analysis*

On combustion, the crystals left a lavender-coloured ash which proved to be cobalt phosphate (Smith 1948*b*). Colorimetric estimations of cobalt with  $\alpha$ -nitroso- $\beta$ -naphthol or  $\beta$ -nitroso- $\alpha$ -naphthol indicated 4.0 % of cobalt in several batches of crystals dried *in vacuo* at 56° C. If each molecule contains one atom of cobalt, the molecular weight of the compound must thus be about 1500. This is in good agreement with the value of 1360 to 1575 calculated by Dr Hodgkin for the dry material from X-ray crystallography data. (The uncertainty is due to doubts about the state of hydration of the crystals used for these measurements.)

The molecular weight by the X-ray method could be a submultiple of this value, whereas that obtained by chemical analysis could be a multiple; thus taken together the two methods fix the molecular weight at about 1500, which means that only one atom of cobalt is present.

The value does not agree with that of about 3000 found by the diffusion method. The discrepancy may be due to errors inherent in the latter method, or to the use of

impure material, but it is also possible that the factor associates in solution either with itself or with peptide impurities.

The material proved difficult to burn, and replicate micro-analyses did not agree well. However, this may be due in part to variable small amounts of impurities adsorbed on the crystals, since the three analyses recorded in table 2 were performed on different samples. Colorimetric determinations of phosphorus, after decomposition by acid digestion, fusion with sodium peroxide or heating with nitric acid in a sealed tube, gave particularly erratic results, but it now appears that the molecule contains only one atom of phosphorus, and not three as suggested in a preliminary note (Smith 1948*b*). Shortage of material precluded additional analyses. The third analysis indicates a formula  $C_{63}H_{97}O_{20}N_{14}PCo$ , but this is put forward very tentatively at the present time.

TABLE 2. ANALYSES OF ANTI-PERNICIOUS ANAEMIA FACTOR

batch no.	LX 7 (%)	LX 11 (%)	LX 17 (%)
loss on drying	not determined	8.04	16.95, 17.19
on dry matter:			
carbon	51.42	51.22	51.69
hydrogen	7.70	8.55	6.70
nitrogen	12.60	13.23	13.55
ash	11.08	9.80	12.39
cobalt	4.0	4.0	4.0
phosphorus	not determined	results in- consistent	2.09, 2.16

The first two analyses were carried out by Miss H. King, the third was kindly made by Dr E. T. Manser (Zurich).

The first two cobalt determinations were made by Mr E. G. Tomich by the  $\alpha$ -nitroso- $\beta$ -naphthol colorimetric method; the third was made by Dr S. Ball using  $\beta$ -nitroso- $\alpha$ -naphthol. The phosphorus determinations were made by Mr S. Trippett at the University of Cambridge.

#### *Stability of anti-pernicious anaemia factor*

The factor is altered chemically by mild treatments. Exposure of dilute solutions of incompletely purified material to daylight slowly altered the colour from pink to orange. After 12 days the pigment was found to be strongly adsorbed on silica from aqueous solution, and there was no evidence that any of the original lightly adsorbed red pigment remained.

Later, a dilute solution of the crystalline material (1.25  $\mu$ g./ml.), after exposure to winter daylight for 9 days, was found to have lost 60% of its initial microbiological activity; the activity of another portion of the same solution left in the dark was unchanged.

We are indebted to Professor D. Keilin for drawing our attention to the action of sodium hydrosulphite. Addition of a very dilute solution at about pH 8 causes a change in colour to orange, and the absorption bands disappear. This change can be reversed on shaking with air, provided no great excess of hydrosulphite is used. Hydrogen peroxide slowly bleaches the red pigment to orange, then to light yellow.

Cold dilute acid and alkali and heating at neutral pH slowly inactivate the factor. There is some loss of colour and a fall in the intensity of the ultra-violet absorption band at  $362\text{ m}\mu$ , particularly in alkaline solution. The loss of microbiological activity is, however, much greater, indicating that the inactivated material is still coloured. Our microbiological assay results are similar to those reported by Rickes *et al.* (1948b).

Solutions initially containing about  $0.1\text{ mg./ml.}$  lost activity as follows:

12 days at room temperature at pH 11.7: 91 % destruction,

32 days at room temperature at pH 1.9: 96 % destruction,

21 hours at  $90^\circ\text{C}$  at pH 7: 75 % destruction.

Solutions containing  $0.1$  to  $0.2\text{ }\mu\text{g./ml.}$  showed no loss of activity when kept in the dark at room temperature for 6 days in water or in ethanol, butanol or acetone each containing 10 % of water, or in water-saturated phenol for 11 days. A neutral aqueous solution containing  $1.25\text{ }\mu\text{g./ml.}$  showed no loss of activity during 2 months at  $37^\circ\text{C}$ .

#### *Acid hydrolysis*

Using incompletely purified material we were able to show that the red pigment undergoes some chemical change on hydrolysis with hot dilute acid. Thus on continued boiling with *N*-hydrochloric acid, the red colour became extractable to an increasing degree by *n*-butanol; the reaction appeared complete after about  $1\frac{1}{4}$  hr. Hydrolysis with *N*-sulphuric acid was appreciably slower, about 4 hr. at the boiling-point being required before the whole of the colour became extractable by butanol. The crystalline compound behaved in the same way. The red degradation product appeared to be a fairly strong acid, as it could be removed from the butanol extract by shaking with sodium bicarbonate solution. This acid was accompanied in some experiments by variable amounts of a substantially neutral red product not extractable from butanol with alkali. Neither product is the same as that formed on inactivation with cold dilute acid or alkali, for this is not readily extractable by butanol from acid solutions. Further chemical work is in progress, and will be reported separately in due course.

#### *Radioactive tracer studies*

We have explored the possibility of labelling the anti-pernicious anaemia factor with radioactive cobalt or phosphorus by direct chemical exchange.

The first experiments were carried out with radioactive phosphorus prepared in the GLEEP (Graphite Low Energy Experimental Pile) at the Atomic Energy Research Establishment, Harwell. In each experiment  $50\text{ }\mu\text{g.}$  of crystalline anti-pernicious anaemia factor were used, together with  $3\text{ }\mu\text{g.}$  of carrier phosphorus and  $1\text{ }\mu\text{c.}$  of  $^{32}\text{P}$ , both in the form of sodium phosphate, in a total volume of  $0.25\text{ ml.}$  of water. After the period allowed for exchange, the solution was diluted and the anti-pernicious anaemia factor was separated from ionized phosphate by extraction with phenol. The phenol layer was washed several times with a dilute acidified solution of sodium phosphate; then water was added and the phenol removed with ether. The radioactivity was measured in a jacketed solution Geiger-Müller counter (Veall 1948). No significant exchange could be induced by standing

at room temperature for 2 hr. in  $N/10$ -acid or alkali, by boiling for 1 hr. at neutral pH or by incubating with fresh rabbit or human liver.

Similar experiments were carried out with radioactive cobalt. For preliminary experiments we used  $^{56+58}\text{Co}$  made by deuteron bombardment of iron in the Berkeley Cyclotron. Later we used  $^{58+60}\text{Co}$  made by neutron bombardment of nickel nitrate in the GLEEP. The cobalt was extracted by the thiocyanate method.

The exchange experiments were conducted similarly to those described with  $^{32}\text{P}$ , except that the phenol extracts were washed with dilute acidified cobalt chloride solution. As before, no significant exchange could be induced under acid or alkaline conditions, by boiling at neutral pH or by incubation with fresh rat liver.

Since the anti-pernicious anaemia factor is presumably a cobaltic co-ordination compound, exchange with cobaltous ion would also involve oxidation. It therefore seemed appropriate to determine whether exchange occurred with another cobaltic complex. For this experiment  $^{60}\text{Co}$  from the Oak Ridge pile was used, and hexammino-cobaltic chloride (luteo) was prepared with a specific activity of  $0.47 \mu\text{c./mg.}$  0.5 mg. of this compound and 0.1 mg. of the anti-pernicious anaemia compound in 0.73 ml. of water at pH 5.5 were left at room temperature for 24 hr. Paper chromatography separated the red spot due to the factor and the lower yellow one due to the luteo compound. After repeating this separation the red spot had negligible radioactivity corresponding with about 0.01 % exchange.

In the light of exchange experiments with cobalt complexes by Flagg (1941) and with nickel complexes by Johnson & Hall (1948), it can be concluded that the cobalt and phosphorus are firmly bound in co-ordinate linkage in the molecule of the anti-pernicious anaemia factor.

Our thanks are due to Dr H. Seligman and others of the Atomic Energy Research Establishment, Harwell, and to Dr A. S. McFarlane and others of the National Institute for Medical Research, Hampstead, for helpful advice and for making available the isotopes used in this work.

In view of these (not unexpected) negative results, it will be necessary to resort to biological synthesis. A series of experiments is in progress in which radioactive cobalt is being administered to animals of various species. It has been shown that 'animal protein factor' can be produced by various micro-organisms, and that it is probably identical with vitamin  $B_{12}$  (Stokstad, Page, Pierce, Franklin, Jukes, Heinle, Epstein & Welch 1948; Cuthbertson & Smith 1949). We are therefore also attempting to prepare the labelled factor by incorporation of radioactive cobalt in suitable media on which are grown vitamin  $B_{12}$ -producing organisms.

#### *Clinical data*

Details of clinical tests carried out to follow the progress of fractionation of liver extracts, and the determination of a dosage-response curve with the crystalline factor, will be published separately by Dr Ungley. According to his results it would appear, as a conclusion from a considerable number of cases, that the minimum effective single dose to induce an optimal response in a case of pernicious anaemia is  $10 \mu\text{g.}$  or possibly even only  $8 \mu\text{g.}$  of the crystalline factor. The daily requirement can be put at about  $0.5 \mu\text{g.}$ , so that the anti-pernicious anaemia factor is more

potent than any known vitamin or hormone. Pteroylglutamic acid (folic acid) given in doses some 10,000 times as great will induce remission of the haematological symptoms of pernicious anaemia; so also will thymine in extremely large doses, though not thymidine (Ungley 1949*a*). Pteroylglutamic acid, however, will not reverse, and may possibly precipitate, the neurological sequelae of pernicious anaemia. In contrast, crystalline anti-pernicious anaemia factor will check and to some extent reverse these changes to the same degree that crude-liver extract will. This claim rests not only upon the single case reported by Berk, Denny-Brown, Finland & Castle (1948), Dr Ungley's three cases treated with incompletely purified material (Smith 1948*a*), the three cases reported by Bethell, Meyers & Neligh (1948) and the six cases reported by Hall & Campbell (1948), but also upon eight cases maintained by Dr Ungley for nearly a year on the crystalline factor (Ungley 1949*b*). This substance has also been found effective against tropical macrocytic anaemia in India (Patel 1948).

It thus appears that all the well-defined conditions in which liver extracts are effective respond equally well to the crystalline anti-pernicious anaemia factor. It seems probable that this factor, together with our other red factor, are the only effective anti-anaemic substances present in refined liver extract, though this cannot be stated with certainty until these factors have been isolated in more nearly quantitative yields.

#### APPENDIX. CRYSTALLOGRAPHIC MEASUREMENTS ON THE ANTI-PERNICIOUS ANAEMIA FACTOR

BY DOROTHY HODGKIN, F.R.S., M. W. PORTER AND R. C. SPILLER  
*University of Oxford*

Since May 1948, five different samples of crystalline anti-pernicious anaemia factor have been examined in Oxford by various crystallographic techniques. These all, from their general characteristics, particularly the intensities of a number of X-ray reflexions, contain the same molecular structure. But the different samples have shown small variations in unit-cell dimensions and in crystal habit, which are probably due mainly to differences in solvent content, combined with traces of different impurities. Small changes in, for example, some side chain in the molecule, might also conceivably contribute to these effects.

The crystals, as grown both from water and from aqueous acetone, are dark red and show marked pleochroism. They vary in habit from long thin needles to short thick prisms on which different crystal faces appear (figures A 1 and A 2). They all contain solvent, probably water, of crystallization, part at least of which they lose slowly on exposure to the air. Crystals kept in their mother-liquor are transparent and show beautifully clear reflecting faces; they give sharp X-ray reflexions extending to spacings of  $1.1 \text{ \AA}$ . On removal from the liquid they tend to crack and to become opaque; the faces are distorted and the X-ray reflexions become first multiple, and then blurred, corresponding to the presence of disorder within the dried crystal structure. But the rate of loss of solvent appears to vary both with the

size of crystals and with the different samples studied. Individual air-dried crystals, for example, have been observed which have given good and sharp X-ray reflexions some weeks after exposure to the atmosphere.

Two principal series of measurements have been carried out. The first was concentrated on samples received in June, July and August, the second on a much larger preparation (about 8 mg.) which included a number of crystals up to  $\frac{1}{2}$  mm.<sup>3</sup> in size and which was received in October 1948. The data recorded on the different samples may be summarized as follows:

### *Crystal morphology*

The crystals show two distinct varieties of crystal habit which are drawn in figures A 1 and A 2.

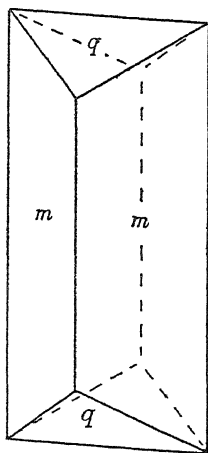


FIGURE A 1

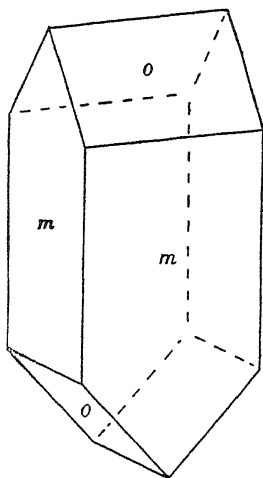


FIGURE A 2

Those belonging to the first variety commonly develop as needles, markedly elongated along  $c$ , bounded by  $m$   $\{110\}$  with  $q$   $\{011\}$  very small. All the earlier samples showed this particular face development; the crystals measured goniometrically were four of size  $\frac{1}{2} \times \frac{1}{6} \times \frac{1}{6}$  mm. chosen from the August sample. The reflexions from these crystals gave double or multiple images of the signal, and some of the crystals appear to be distorted.

The second variety, figure A 2, is that shown by the October sample. These crystals are usually short prisms. As they grow on slides from solution in water, they frequently lie on one of the prism faces and then appear terminated at one end by two edges at  $45^\circ$  to one another,  $(111)$ ,  $(\bar{1}\bar{1}1)$ , and at the other by a single edge at  $90^\circ$  to the prism axis.

Goniometric measurements give the following data:

Crystal system, orthorhombic. Class bisphenoidal (222):

$$a:b:c = 1.142:1:0.752$$

(cf.  $a:b:c = 1.140:1:0.751$  from X-ray data on crystal 1.)

Forms:  $m\{110\}$ ,  $q\{011\}$ ,  $o\{111\}$ , or  $o'\{\bar{1}\bar{1}1\}$ .

Habit:  $m, q$ ; prismatic [001] (see figure A 1).  $m, o$  or  $m, o'$ ; prismatic [001] (see figure A 2).

$$\begin{aligned}\text{Angles:} \quad qq &= (011):(0\bar{1}1) = 73^\circ 54', \\ mm &= (110):(1\bar{1}0) = 97^\circ 34', \\ om &= (111):(110) = 45^\circ 0' .\end{aligned}$$

Barker setting: transformation equation  $010/\bar{1}00/001$ ,  $m = (1\bar{1}0)$ ,  $q = (101)$ ,  $o = (1\bar{1}1)$ ,  $o' = (\bar{1}11)$ .

Classification angles:  $36^\circ 57'$ ,  $41^\circ 13'$ ,  $56^\circ 37'$ .

### Optics

The refractive indices of air-dry crystals of vitamin B<sub>12</sub> were recorded by Rickes *et al.* (1948a) as  $X = 1.616$ ,  $Y = 1.652$ ,  $Z = 1.6645$ . With these figures the measurements carried out in Oxford are in general agreement. These measurements were made on crystals of the October sample of anti-pernicious anaemia factor recrystallized from water in drops on a slide. The crystals are, as mentioned above, optically negative, but the measured values of the refractive indices appear to be slightly lower;  $Z$  nearly equal to 1.656 and  $X = 1.591 (\pm 0.005)$ . The orientation of the optic axes to the crystal axes is  $X//a$ ,  $Y//b$ ,  $Z//c$ . Further, the crystals are markedly pleochroic with  $Z$  and  $Y$  strong carmine red, and  $X$  paler red to almost colourless. Since they tend to lie with various orientations of the optic axes to the slide, some crystals usually appear obviously pleochroic and others not.

Some difficulty was experienced in making measurements of the refractive indices, particularly of the earlier preparations, owing to the tendency of some of the material to separate in an amorphous form surrounding the crystals when these were grown on slides.

### X-ray data

X-ray photographs were taken of crystals mounted about all three crystallographic axes using both copper and chromium X-radiation.\* These included oscillation and Weissenberg photographs; one powder photograph also was recorded (see plate 27 and table A 2). The wet crystals were grown for X-ray photography by slow evaporation of drops of solution enclosed in fine-walled Hysil glass capillaries. The tubes were sealed as soon as large enough crystals had developed and before all liquid had evaporated. The unit-cell dimensions observed on the different crystals photographed are given in table A 1. With the exception of nos. 1 and 2, these cell

TABLE A 1

	date of photography	$a$	$b$	$c$
1. air-dried crystal	June 1948	24.35	21.36	16.04
2. wet crystal, October sample	January 1949	25.51	22.52	15.92
3. air-dried crystal, October sample	December 1948	24.1	21.3	16.14
4. air-dried crystal, October sample	January 1949	24.4	21.2	16.25
5. air-dried crystal, August sample	December 1948	23.7	21.45	15.9

\* We are indebted for the loan of a chromium target tube to the Atomic Energy Research Establishment and Dr Thewlis.

dimensions could only be measured approximately owing to the distortion of the dry crystals. Nevertheless, the differences between them appear to be real.

The crystal space group in all cases is  $P2_12_12_1$ , for which the number of units required is 4. The density of the air-dried crystals (1) was  $1.338 \pm 0.002$ ; that of the wet crystals (2) is less certain, but appeared to be very much the same, 1.34. Both were measured by flotation in *o*-dichlorobenzene and  $\alpha$ -bromonaphthalene mixtures.

The best measurements from the point of view of molecular weight determination were those carried out on crystals no. 1, which were small, well-formed, clear needles. The weight of the asymmetric unit in this crystal structure can be calculated as  $1680 \pm 30$ , and this represents an upper limit for the molecular weight of the anti-pernicious anaemia factor itself. The exact value of the molecular weight depends on the weight of solvent present in the crystals. Analyses carried out shortly after the X-ray measurements were made gave a value of 8%. This would give a figure of 1545 as the possible weight of the active molecule. But the figure is certainly doubtful since the solvent loss occurs gradually, and we do not know that the crystals dried for analysis were in the same state of dryness as those examined by X-rays. The figure of 17% solvent loss recorded later, combined with unit-cell dimensions no. 1, would give a lower figure, 1392, for the molecular weight. But again, this solvent loss might be derived from less dry crystals. Combined with the cell dimensions of the wet crystals, for example, no. 2, it would lead to a calculated weight of 1530.

TABLE A2. SPACING AND INTENSITIES OF LINES ON POWDER PHOTOGRAPH\*  
OF ANTI-PERNICIOUS ANAEMIA FACTOR, AUGUST SAMPLE

<i>d</i>	relative intensity	<i>d</i>	relative intensity
16.07	1.00	5.02	0.20
12.81	1.00	4.93	0.20
12.46	0.40	4.72	0.20
11.30	0.70	4.45	0.50
9.72	0.70	4.29	0.18
8.87	0.50	4.16	0.20
8.16	0.10	4.02	0.30
7.55	0.30	3.88	0.30
7.20	0.30	3.74	0.26
6.76	0.50	3.58	0.24
6.44	0.35	3.41	0.46
6.07	0.18	3.22	0.30
5.87	0.20	3.10	0.18
5.36	0.30	3.04	0.14

\* Taken by Mrs A. F. Joseph.

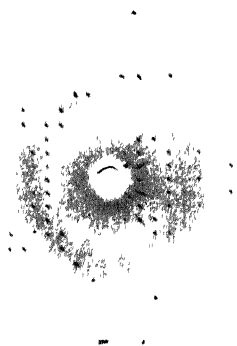
In the circumstances, the X-ray data can only limit the molecular weight as lying probably between 1360 and 1575. It might also be some submultiple of these figures.

Two points about the crystallographic data in general are perhaps worth mention. First, the phenomenon shown here, of loss of solvent from the crystals combined with the development of crystal disorder, has many parallels, particularly among proteins. Here the changes in cell dimensions are relatively small, but similar in

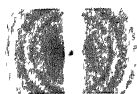




*a.* 15° oscillation photograph of dry crystals of October sample, rotation axis [100], beam direction (010).



*b.* 15° oscillation photograph of wet crystals of October sample, rotation axis [100], beam direction (010).



*c.* Powder photograph of August sample, Cu radiation, camera diameter 114.2 mm.

X-ray photographs of anti-pernicious anaemia factor.

(Facing p. 612)



magnitude to those observed among certain peptides, derivatives of gramicidin S, for example. In such systems, the stability of the various wet and dry forms can be very much affected by the presence of traces of impurities. Secondly, the marked pleochroism of the crystals suggests the arrangement of colour-bearing groups in a plane surrounding the cobalt, the octahedral co-ordination being completed by other groups, possibly water molecules. The orientation of this plane in the crystal is roughly perpendicular to the *a*-axis.

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# The annelid phosphagen: with a note on phosphagen in Echinodermata and Protochordata\*

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A new, arginine phosphate-like phosphagen is present in annelid and gephyrean worms.

A second phosphagen, possibly identical with creatine phosphate, has also been found in certain annelids but not so far in gephyreans. This second phosphagen sometimes co-exists with the first, but is found alone in some species.

The distribution of these two phosphagens does not appear to be correlated with physiological activity or with environmental factors.

Arginine could not be isolated from either of two annelids or one gephyrean species; a new base is, however, present in the guanidine fraction and has been isolated as the picrate. It has not yet been identified.

The significance of these observations is discussed in relation to the taxonomic status of the Annelida and Gephyrea. It is indicated that there exists a close relationship between these groups.

The new 'annelid phosphagen' is, apparently, confined to the Annelida and Gephyrea, which are thus chemically distinguishable from the Arthropoda and Mollusca.

Some new data are presented concerning the distribution of arginine and creatine phosphates in Echinodermata and Hemichordata, and the evidence concerning these two groups is reviewed with special reference to the echinoderm-hemichordate theory of vertebrate ancestry.

It is concluded that existing data support this theory, and that the new information concerning the phosphagens of the annelids serves to emphasize the wide divergence that exists between the segmented invertebrates and the true Chordata.

## INTRODUCTION

It has been generally assumed hitherto that the creatine phosphate characteristic of vertebrate muscle is always replaced by arginine phosphate in the muscles of invertebrates. This assumption has rested largely upon the isolation of arginine from representative members of most of the invertebrate phyla (see the reviews of Kutscher & Ackermann 1926, 1933, 1936). In addition, there have been many demonstrations, especially by Arnold & Luck (1933), of the occurrence in invertebrate muscles of a substance that is susceptible to the action of arginase, a highly specific enzyme (Baldwin 1936), with formation of urea.

A valuable accessory source of evidence lies in the fact that, whereas the acid hydrolysis of creatine phosphate is greatly accelerated by the addition of molybdate ions, that of arginine phosphate is much retarded (Meyerhof & Lohmann 1928). Needham, Needham, Baldwin & J. Yudkin (1932), relying on the qualitative effect

\* A preliminary account of a part of this work has already appeared (Baldwin & Yudkin 1948).

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of molybdate on the rate of hydrolysis of the two phosphagens as a diagnostic characteristic, carried out a broad comparative study of invertebrate animals, but, with certain notable exceptions among the Echinodermata, to which reference is made in the note to the present paper, found no reason to think that any phosphagen other than arginine phosphate occurs among invertebrates. In *Sipunculus* sp., a gephyrean, Meyerhof (1928) had previously demonstrated the presence of a phosphagen with the properties of arginine phosphate, the basic component of which was, moreover, hydrolyzed by arginase. Confirmation of this observation was furnished by Needham *et al.* (1932), who found in *S. nudus* a compound which behaved towards molybdate like arginine phosphate, but they did not characterize it further. A similar compound was also detected in three marine polychaete worms, *Sabellaria alveolata*, *Spirographis brevispira* and *Nereis diversicolor*. Later, Borsuk, Kreps & Verjinskaya (1933) found what appeared to be arginine phosphate in *Arenicola marina*.

But Arnold & Luck (1933), using a method based on the use of arginase and xanthidrol, were unable to find any trace of arginine in any of the five polychaete and three gephyrean species they studied, nor could Kurtz & Luck (1937-8) find any evidence for the identity of the annelid phosphagen with arginine phosphate. This appears as clear-cut evidence, in disagreement with previous interpretations, that arginine phosphate is not present in annelids, including gephyreans.

At the same time and using the same method, Arnold & Luck (1933) demonstrated the presence of arginine in numerous other invertebrate species including *Lumbricus* sp., an oligochaete, thus confirming the work of Kutscher & Ackermann (1931), who succeeded in isolating arginine from *L. terrestris*. In both cases, however, the quantities of arginine found were relatively very small. More recently Greenwald (1946) has shown that the reproductive organs of certain marine annelids contain a material that is strongly chromogenic in the Jaffé reaction and may possibly be creatine.

Through the generosity of the Lalor Foundation we had an opportunity to work at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer of 1948 and decided to undertake a more detailed study of the phosphagen of marine annelids and gephyreans than had previously been attempted. Some further experiments were later carried out at the Plymouth Laboratory.

#### METHODS

Freshly collected animal material was used whenever possible and was cooled to 0° C before use. Some of the larger species were gutted before being extracted; the smaller species were extracted whole. When the animals had been immobilized by cooling, the weighed and thoroughly chilled material was ground with sand or powdered glass and extracted in an ice-cold mortar with ice-cold 8 % trichloroacetic acid, usually in the proportion of 5 ml./g. of tissue. After filtration with the aid of kieselguhr through an ice-cold Gooch crucible, the filtrates were kept at about -2° C and worked up as rapidly as possible.

As a rule, 4 ml. samples of the filtrate were taken for analysis. Enough 40 % NaOH was cautiously added to make the fluid pink to phenolphthalein and 1 ml. of the

$\text{Ca}(\text{OH})_2\text{-CaCl}_2$  reagent of Fiske & Subbarow (1929) was added. After mixing, the whole was allowed to stand in ice for 10 min. and then centrifuged. Phosphagen was estimated in the clear centrifugate and inorganic phosphate in the precipitate. Estimations of the other acid-soluble phosphorus compounds were not carried out.

The precipitate, dissolved in a minimum quantity of  $2N \text{ H}_2\text{SO}_4$ , was quantitatively transferred to a volumetric flask and treated with the Fiske & Subbarow (1929) colour reagents, made up to the mark and allowed to stand for 10 to 15 min. before colorimetry. We used a Klett-Summerson photoelectric colorimeter in conjunction with the usual red filter (no. 66) throughout the work at Woods Hole. The estimations at Plymouth were carried out with a photoelectric colorimeter designed and built by Harvey (1948) with a Chance OR2 filter.

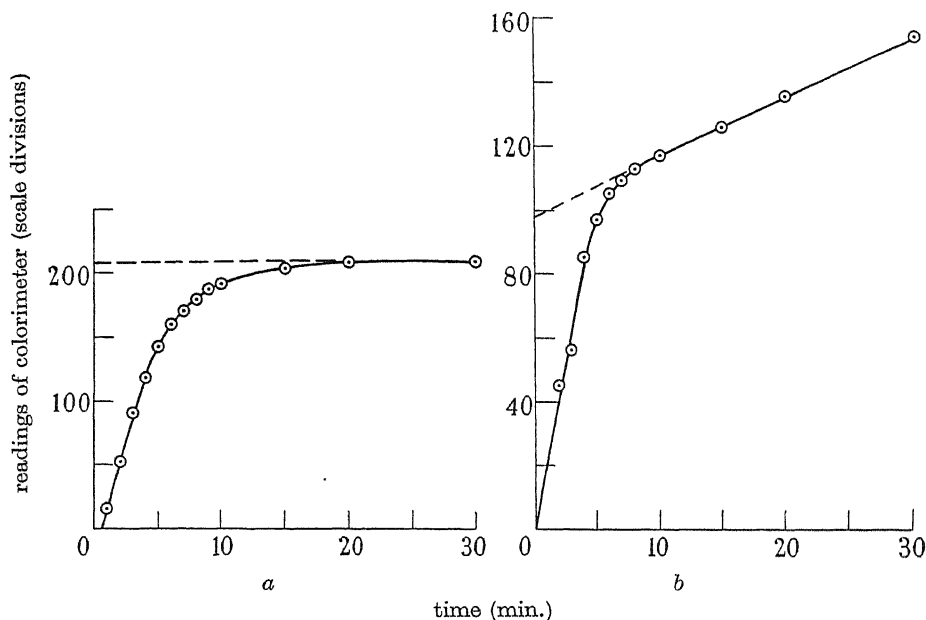


FIGURE 1. Hydrolysis of phosphagen of *a*, *Orbinia ornata*; *b*, *Neanthes virens*. 0.25 % ammonium molybdate and  $0.5N \text{ H}_2\text{SO}_4$  present; room temperature.

For the phosphagen estimations the filtrate was ordinarily divided into two portions. To one, trichloroacetic acid was added to give a 2 % solution; this was incubated for 60 sec. in a boiling water-bath to hydrolyze the total phosphagen (cf. Lohmann 1936). After cooling, the usual colour reagents were added and the total phosphagen P estimated colorimetrically. The second portion was treated with the colour reagents directly, made up to the mark, mixed and placed in the colorimeter as rapidly as possible, usually within 1 min. of addition of the reagents; control experiments showed that 95 % of the full colour due to inorganic P was developed within 1 min. under our conditions. Hydrolysis of the phosphagen was followed by taking serial readings over a period of at least 30 min. and at 1 min. intervals for the first 5 to 10 min. These readings were plotted against time.

In some cases we found that two labile phosphagen-like compounds were present, one behaving like creatine phosphate and the other like its arginine analogue. The

creatine phosphate-like substance ('CP') was rapidly decomposed under these conditions, hydrolysis being complete within 15 to 20 min. (see figure 1*a*). The arginine phosphate-like compound ('AP') was hydrolyzed much more slowly however, so that, when both were present in the same extract, 'CP' could be estimated by backward extrapolation to zero time (see figure 1*b*), as in the extrapolation procedure devised by Eggleton & Eggleton (1929). As we had the advantage of a highly sensitive, direct-reading instrument our results are probably more accurate than could have been had by the Eggletons' original technique.

In most of our experiments, samples of the residual trichloroacetic acid filtrates were allowed to warm up so that the phosphagens might decompose. In other cases we used the centrifugates after calcium precipitation and allowed the phosphagens to decompose. The Sakaguchi test for arginine and the Vosges-Proskauer reaction (after Walpole 1911) for creatine were carried out on these samples; although neither of these reactions is very specific, useful indications were nevertheless obtained.

In addition to the general procedures described in the preceding paragraphs, special techniques were employed for certain purposes and are described in the appropriate context later in this paper.

#### DISTRIBUTION STUDIES

In all, twenty-four species of marine annelids and geophyreae were examined, and the analytical results obtained are summarized in table 1, together with some data taken from earlier work in this field. Two typical hydrolysis curves are shown in figure 1, the first (*a*) for extracts of the polychaete *Orbinia ornata*, in which only 'CP' was present, and the second (*b*) for another polychaete, *Neanthes virens*, in which both substances were present side by side. The species examined at Woods Hole are named in accordance with Pratt (1935), those studied at Plymouth in accordance with the Plymouth Marine Fauna list (1931).

#### PROPERTIES OF THE CREATINE PHOSPHATE-LIKE COMPOUND ('CP')

As table 1 shows, positive Vosges-Proskauer reactions were obtained in practically every case in which 'CP' was present in the extracts. It seemed worth while, therefore, to take further steps towards the identification of the compound. We accordingly took the creatine phosphate present in trichloroacetic acid extracts of the sartorius muscles of the frog, *Rana pipiens*, as a reference standard and put it through the extrapolation procedure in exactly the same manner as the worm extracts. The resulting curve is plotted, on a percentage basis, in figure 2, together with the points observed with extracts of those worms in which 'CP' was found, the 100 % value for which was determined by backward extrapolation in the usual manner.

It will be observed that there is, in general, reasonable agreement between the experimental points and the reference curve. Taken together with the positive Vosges-Proskauer tests and the observations of Greenwald (1946) previously mentioned, it seems reasonable to conclude, at any rate provisionally, that 'CP'

TABLE 1. DISTRIBUTION OF 'AP' AND 'CP' IN ANNELIDS AND GEOPHYREANS

animal	mg. P/100 g.					Walpole test	Sakaguchi test	references
	inorganic	'CP'	'AP'	total	total phosphagen			
ANNELIDA								
Polychaeta								
<i>Amphitrite johnstoni</i> (whole)*	19.6	0.0	11.7	31.3	11.7	—	+	
<i>A. ornata</i> (body wall)	18.2	0.0	19.7	37.9	19.7	o	o	
<i>A. ornata</i> (whole)	8.8	0.0	10.2	19.0	10.2	o	o	
<i>Arabella tricolor</i> (whole)	35.9	0.3	3.2	39.4	3.5	+	—	
<i>Arenicola marina</i> (body wall)*	11.1	0.0	26.9	38.1	26.9	—	+	
<i>A. marina</i> (body wall)*	12.5	0.0	22.5	35.0	22.5	+	+	
<i>Branchiomma vesiculosum</i> (whole)*	17.7	2.2	3.8	23.7	6.0	+	+	
<i>Chaetopterus varipodatus</i> (whole)	24.1	1.7	0.0	25.8	1.7	+	+	
<i>Cirratulus grandis</i> (whole)	11.4	0.0	3.9	15.3	3.9	—	±	
<i>Cistenides gouldii</i> (whole)	10.4	1.0	2.7	14.1	3.7	—	+	
<i>Clymenella torquata</i> (whole)	37.1	0.0	6.7	43.8	6.7	—	+	
<i>Clymenella torquata</i> (whole)	22.7	8.0	0.0	30.7	8.0	—	+	
<i>Diopatra cuprea</i> (whole)	5.6	0.0	7.0	12.6	7.0	+	+	
<i>Enoplobranchius sanguinea</i> (whole)	58.5	9.7	0.0	68.2	9.7	+	—	
<i>Glycera dibranchiata</i> (whole)	48.0	25.0	0.0	73.0	25.0	+	—	
<i>G. dibranchiata</i> (whole)	29.4	1.8	3.8	35.0	5.6	+	+	
<i>Lepadometria commensalis</i> (whole)	16.2	14.0	0.0	30.2	14.0	+	+	
<i>Lumbrineris</i> sp. (whole)	15.3	0.0	7.2	22.5	7.2	—	+	
<i>Maldane uveolata</i> (whole)	30.1	29.6	26.4	86.1	56.0	±	+	
<i>Neanthes virens</i> (whole)	30.8	19.8	48.5	99.1	68.3	+	o	
<i>N. virens</i> (whole)	27.6	27.5	67.5	122.6	95.0	+	+	
<i>N. virens</i> (whole)	28.2	39.2	41.5	108.9	80.7	+	+	
<i>N. virens</i> (gutted)	24.0	0.0	5.1	29.1	5.1	—	+	
<i>Nereis cultrifera</i> (whole)*	22.6	0.0	10.2	32.8	10.2	—	+	a
<i>N. diversicolor</i> (whole)*	21.0	2.5	13.7	37.2	16.2	—	o	a
<i>N. diversicolor</i> (whole)	21.5	0.0	13.7	35.2	13.7	—	o	
<i>N. diversicolor</i> (whole)	10.0	12.9	0.0	22.9	12.9	+	±	
<i>Orbinia ornata</i> (whole)	17.2	0.0	19.2	36.4	19.2	—	+	
<i>Sabella palmata</i> (whole)	21.6	0.0	17.3	38.9	17.3	—	+	
<i>Sabella pavonia</i> (whole)*	27.8	0.0	8.8	36.6	8.8	o	o	
<i>Sabellaria alveolata</i> (whole)	9.9	0.0	17.2	27.1	17.2	o	o	
<i>Spirographis brevispira</i> (whole)	24.1	2.8	5.6	32.5	8.4	+	+	
<i>Sthenelais ledgii</i> (whole)								
Oligochaeta								
<i>Lumbricus terrestris</i> (whole)	13.6	0.0	0.0	13.6	0.0	o	o	
GEOPHYREA								
Spinaculoidea								
<i>Phascolosoma elongatum</i> (body wall)*	32.4	0.0	18.5	51.9	18.5	—	+	
<i>Ph. gouldii</i> (whole)	3.4	0.0	10.8	14.2	10.8	—	+	
<i>Ph. gouldii</i> (body wall)	15.7	0.0	32.1	48.1	32.1	o	o	
<i>Ph. gouldii</i> (body wall)	18.7	0.0	30.6	49.3	30.6	o	o	
<i>Synneulus nudus</i> (body wall)	13.3	0.0	31.9	45.2	31.9	o	o	a
<i>S. sp.</i> (retractor muscle)	42.2	0.0	67.3	109.5	67.3	o	o	b
<i>S. sp.</i> (longitudinal muscle)	56.6	0.0	170.3	226.9	170.3	o	o	b

\* Experiments carried out at Plymouth.

o indicates that the Sakaguchi or Walpole test was not carried out.

Figures in italics refer to specimens that were still alive and active but had been in the laboratory for some days.

References: (a) Data selected from Needham *et al.* (1932). (b) Data recalculated from Meyerliot (1928). All other data from present work.



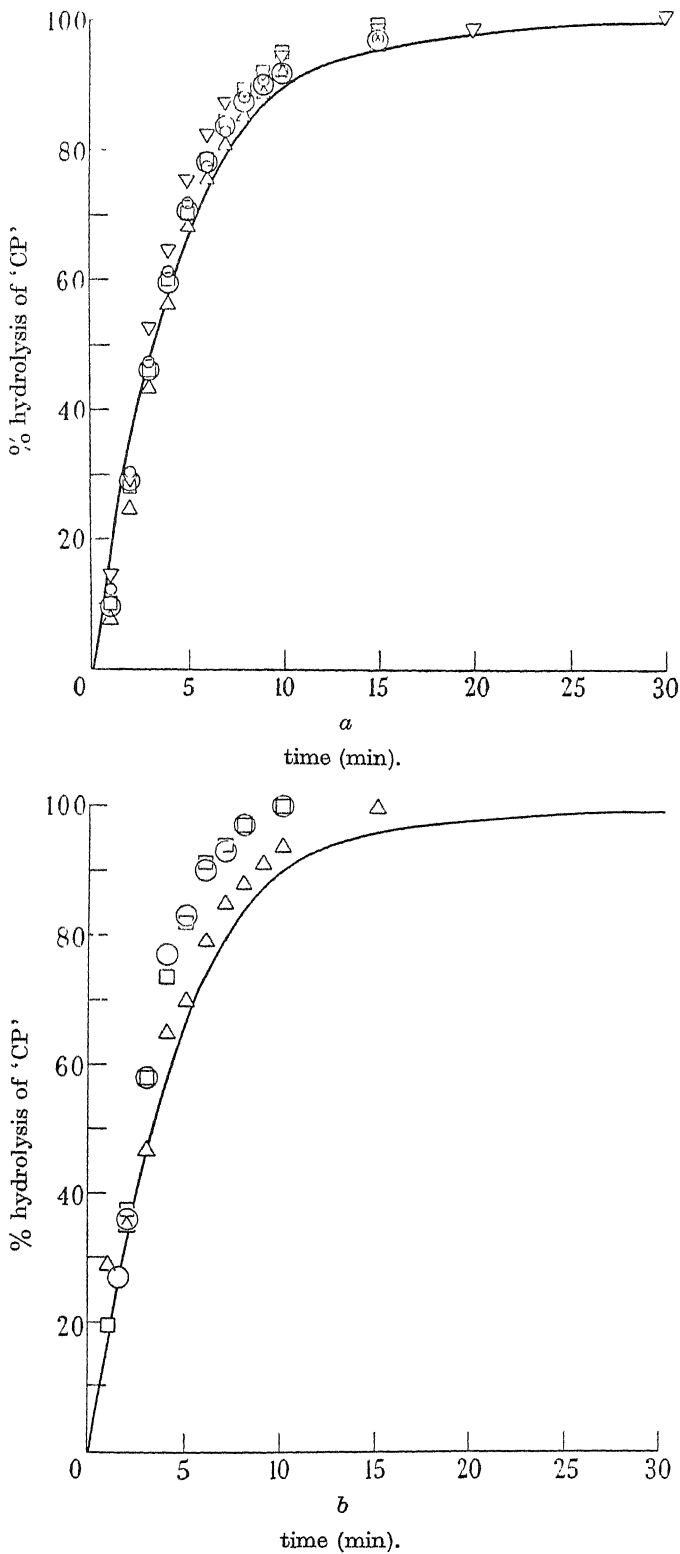


FIGURE 2. Hydrolysis of 'CP' of polychaetes and gephyreans. 0.25 % ammonium molybdate and 0.5 N  $H_2SO_4$  present; room temperature. Full line is the hydrolysis curve of creatine phosphate from *Rana pipiens*. a, species containing 'CP' only: ▽ *Chaetopterus*, ○ *Glycera*, ◊ *Lumbrinereis*, △ *Orbinia*, ◻ *Arabella*. b, species containing 'CP' and 'AP'; 100 % value obtained by extrapolation (see text): ◻ *Lepidometria*, ○ *Neanthes*, △ *Sthlenelais*.

is none other than creatine phosphate. Positive and final demonstration of its identity must, however, await its actual isolation or that of the free base; further work in this direction is projected.

#### PROPERTIES OF THE ARGININE PHOSPHATE-LIKE COMPOUND ('AP')

The Sakaguchi reactions recorded in table 1 were, without exception, far weaker than was to be expected on the assumption that the base of the phosphagen consisted of arginine. In the hope that it might be possible to demonstrate some sharp difference between the 'AP' of our extracts and an authentic sample of arginine phosphate, we carried out a series of determinations of rates of hydrolysis in the presence and absence of molybdate.

For these experiments trichloroacetic acid extracts were prepared as before. 8 ml. samples were treated with 2 ml. of the calcium reagent and centrifuged after standing in ice for 10 min. In each case the centrifugate was neutralized with  $N/10$  HCl and made up to exactly 11 ml. under ice-cold conditions. Total phosphagen P was estimated in the 1 ml. samples remaining after the following experiments had been set up.

Two samples, each of 5 ml., were pipetted into 50 ml. volumetric flasks, each containing about 25 ml. of ice-cold distilled water. One sample now received 5 ml. 2.5 % ammonium molybdate in  $5N$   $H_2SO_4$  and the second 2.5 ml.  $10N$   $H_2SO_4$ . Both were then made up to the mark, still in the cold. One 5 ml. sample was withdrawn from each flask for the estimation of preformed inorganic P. The remaining solutions were rapidly heated to  $38^\circ C$  by immersing the flasks in a boiling water-bath, then securely corked and transferred to the incubator at  $38^\circ C$ . Further 5 ml. samples were taken for P estimations at intervals until more than 80 % of the phosphagen had been hydrolyzed. Finally, the amounts of phosphagen P hydrolyzed were plotted against time (figure 3).

As a reference standard we used the arginine phosphate of the crab, *Libinia emarginata*, a typical crustacean in which we felt it safe to assume that authentic arginine phosphate is present. This assumption was supported by the finding of intense positive Sakaguchi reactions in extracts in which the phosphagen had been allowed to decompose, and by the numerous past demonstrations of the presence of arginine in crustacean material (e.g. in *Crangon vulgaris*, Ackermann & Kutscher, 1906; *Palinurus* sp., Suzuki, Joshimura, Jamakawa & Irie, 1909; 'Grossen Krabbe von Echizen', Suzuki, Inuye & Bharathar 1912; *Astacus fluviatilis*, Kutscher, 1914; *Palinurus japonicus*, Okuda, 1919; *Homarus vulgaris*, Hoppe-Seyler, 1928, 1933). To these isolations of arginine may be added that of arginine phosphate itself from *Astacus fluviatilis* by Meyerhof & Lohmann (1928) and by Lehmann (1935), while evidence of the presence of an arginase-labile compound has been given by Arnold & Luck (1933) for nine further species of Crustacea.

For these experiments we selected *Amphitrite ornata*, a polychaete, and *Phascolosoma gouldii*, a geophyrean, in neither of which was the 'AP' accompanied by any detectable amounts of 'CP'. The hydrolysis curves obtained with the *Libinia* extracts are plotted in figure 3, together with the experimental points obtained

for *Amphitrite* and *Phascolosoma*. Although the points obtained in the presence of molybdate fit the standard curve very well, those in the absence of molybdate fall well below it. It is impossible, however, to regard these differences as conclusive evidence that 'AP' is not identical with arginine phosphate.

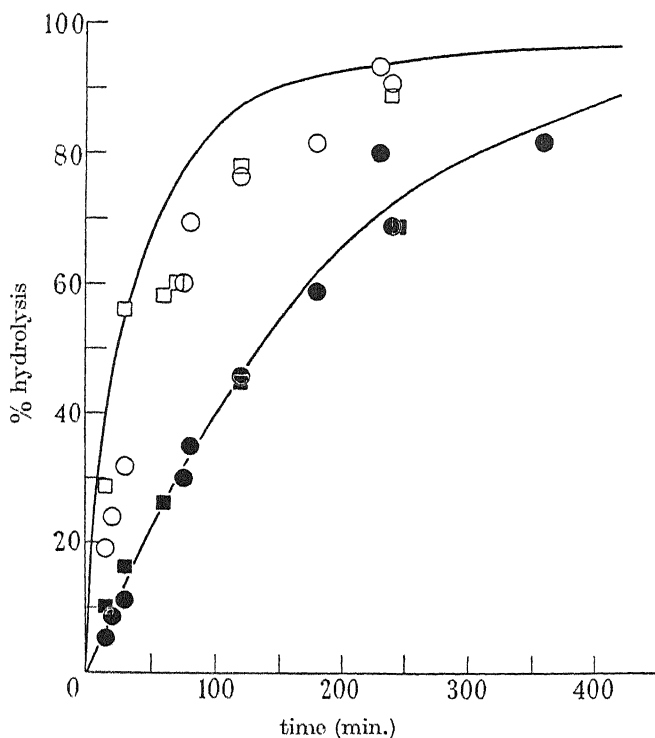


FIGURE 3. Influence of 0.25% ammonium molybdate on acid hydrolysis of arginine phosphate of *Libinia* and 'AP' of *Phascolosoma* and *Amphitrite*. 0.5N  $H_2SO_4$  present throughout; 38° C. *Amphitrite*: ○ without molybdate, ● with molybdate. *Phascolosoma*: □ without molybdate, ■ with molybdate. Full line, *Libinia*, upper line without molybdate, lower line with molybdate.

TABLE 2. TIME TAKEN FOR 50 % HYDROLYSIS OF PHOSPHAGENS

(0.5N H <sub>2</sub> SO <sub>4</sub> ; molybdate 0.25 %; 38° C)				
		molybdate		retardation factor
class	species	absent	present	
Crustacea	<i>Libinia emarginata</i>	28	120	4.3
Polychaeta	<i>Amphitrite ornata</i>	55	135	2.5
	<i>Amphitrite ornata</i>	45	115	2.5
Gephyrea	<i>Phascolosoma gouldii</i>	45	140	3.1

Similarly, equivocal results came out of a consideration of the time taken for 50 % hydrolysis of the phosphagens in presence and absence of molybdate (table 2). The retardation factors found are much smaller in every case than those of 15 to 30 found, for example, by Meyerhof & Lohmann (1928) for the arginine phosphate

of *Astacus fluviatilis*, or that found for the arginine phosphate of fly muscle (*Calliphora*, *Lucilia* spp.) by Baldwin & Needham (1933). Values of this smaller order have, however, been found for the authentic arginine phosphates of *Holothuria tubulosa* (Baldwin & Needham 1937) and *Eledone moschata* (Baldwin 1933*b*; Lohmann 1936). These differences are probably attributable to the crude nature of the extracts under examination (Meyerhof & Lohmann 1928) and to the use of different samples of ammonium molybdate (Lohmann 1936). The difference between the retardation factor of 4.3 for *Libinia* and the average figure of 2.7 for the worms may possibly be significant, but affords no conclusive proof that 'AP' differs from the true arginine phosphate.

We next attempted to settle the matter by direct isolation of the base. *Amphitrite* and *Phascolosoma* were both available in quantity at Woods Hole. We gutted large numbers of both species and obtained two batches of body wall material (127 and 226 g.) of the former and one (95 g.) of the latter. The tissues were comminuted in a Waring Blendor with 5 times their own weight of water; the product was boiled and deproteinized with tannic acid. After cooling and filtration, sulphuric acid was added to give an acid concentration of 5 %  $\text{H}_2\text{SO}_4$  and the nitrogenous bases were precipitated by the addition of 25 % phosphotungstic acid containing 5 %  $\text{H}_2\text{SO}_4$ . The precipitates were washed with 5 %  $\text{H}_2\text{SO}_4$  and brought back to Cambridge, still somewhat moist, for further examination.

After removal of a small excess of tannic acid, the phosphotungstates were decomposed and the bases fractionated by the Kossel-Kutscher silver-baryta method (after Guggenheim 1940). Attention was concentrated on the so-called guanidine fraction, in which any arginine present should have been found. This fraction yielded only an exceedingly weak Sakaguchi reaction and gave no precipitate with flavianic acid. A crystalline precipitate was slowly formed on addition of neutral picric acid; after removal of the picric acid from the product, no Sakaguchi reaction was observable. On account of the small amounts available this picrate has not yet been identified. It explodes on analysis. It is not arginine picrate (m.p. 217 to 218° C) since it melts at 277° C after repeated recrystallization. It differs, moreover, from the picrates of the other guanidine bases at present known to occur in invertebrate materials. The same picrate was obtained from both samples of *Amphitrite* and from *Phascolosoma*; there was no depression of the melting-point when the picrates were mixed together mechanically or after mixed recrystallization.

While it would be premature to conclude that this picrate is that of the base of the new annelid phosphagen it is at least clear the base itself, like 'AP', is present in both the species examined. It is likewise clear that neither species contains arginine itself, and the same may reasonably be assumed to be true of the other polychaetes studied.

Further work is in progress on the isolation and identification of the new picrate using the body wall of *Arenicola marina* as starting material: preliminary examination has revealed the presence of a base which yields a picrate identical with that obtained from the other two species.

## DISCUSSION

Two orders of Chaetopoda are usually recognized, Polychaeta and Oligochaeta. In the former, two phosphagens have now been discovered, 'CP', which may well be identical with creatine phosphate, and 'AP', the base of which is still unknown. Among the species studied at Woods Hole all the free-swimming forms, which have been somewhat arbitrarily classified together under the title of Errantia, contained 'CP', sometimes together with 'AP'. The sedentary forms (Sedentaria), with a single exception in the case of *Chaetopterus*, contained 'AP', sometimes together with 'CP'. To this latter group there might be added *Spirographis brevispira*, and *Sabellaria alveolata*, two tubiculous species which Needham *et al.* (1932) found to contain only an arginine phosphate-like compound. We pointed out this apparent correlation in a preliminary note (Baldwin & Yudkin 1948). But, in some later experiments carried out at Plymouth, while *Arenicola marina*, *Amphitrite johnstoni* and *Sabella pavonina* (Sedentaria) contained only 'AP', *Nereis diversicolor* and *N. cultrifera* (Errantia) similarly contained 'AP' but gave no evidence of containing 'CP'. *Branchiomma vesiculosum* (Sedentaria) contained 'CP' and apparently no 'AP'. The ecological distinction between Errantia and Sedentaria finds no parallel, therefore, in the distribution of the two phosphagens, contrary to our first impression. Particularly striking in this connexion is the fact that while *Nereis diversicolor* (see also Needham *et al.* 1932) and *N. cultrifera* contain only 'AP', the very closely related *Neanthes virens* contains both phosphagens. At present, therefore, the occurrence of these two phosphagens cannot be correlated with the physiological factor of over-all muscular activity, with the ecological factor of habitat or with any other environmental factor; indeed, their distribution appears to be entirely erratic.

Little is known about the phosphagen of the Oligochaeta. The only oligochaete genus so far studied, *Lumbricus*, is known to contain arginine (Kutscher & Ackermann 1931; Arnold & Luck 1933) though the amounts present are small. Creatine phosphate-like substances are absent (Eggleton & Eggleton 1928). In a series of preliminary experiments carried out by one of us (W.H.Y.) it has proved impossible to detect any trace of phosphagen resembling either the arginine or the creatine compound. Whether this may be true of the Oligochaeta in general remains to be seen, but one conclusion is already evident; even in *Lumbricus*, which is known to contain arginine, arginine *phosphate* is absent.

As yet no studies appear to have been made of the other two acknowledged annelid classes, Archiannelida and Hirudinea, but in the meantime we have a certain amount of chemical evidence concerning the Gephyrea (Sipunculoidea and Echiuroidea), the relationship of which to the Annelida is somewhat obscure. 'The groups Sipunculoidea...and Echiuroidea... remain of uncertain status. The alternative to considering them separate phyla is to append them to the Annelida' (Hyman 1940).

There is no evidence for the occurrence among Gephyrea of any phosphagen having the properties of creatine phosphate (*Sipunculus* sp., Meyerhof, 1928; *S. nudus*, Needham *et al.* 1932; *Phascolosoma gouldii* and *P. elongatum*, present

work, see table 1). This, it might be claimed, is evidence that the Gephyrea are, at most, only distantly related to the Chaetopoda, many of which contain 'CP'. There is, however, a good deal of evidence that a substance resembling arginine phosphate is present in gephyrean muscle (Meyerhof, 1928; Needham *et al.* 1932; present work) but, at the same time, little evidence that gephyrean muscle contains arginine. Meyerhof's (1928) evidence, gained by the use of liver arginase and jack-bean extracts, pointed to the presence of arginine in *Sipunculus* sp., whereas Arnold & Luck (1933), using arginase followed by xanthidrol precipitation instead of enzymic hydrolysis of urea formed, could detect no arginine in any of the three gephyrean species (*Physcosoma agassizii*, *Dendrostoma zostericola*, *Urechis caupo*) which they examined. We ourselves could find no arginine in extracts of the body wall of *Phascolosoma gouldii* (p.618). While it is difficult to reconcile Meyerhof's observations with those of Arnold & Luck and ourselves, unless, indeed, *Sipunculus* is an exceptional case, it seems reasonable, considering all the evidence, to conclude that 'AP' is probably a usual constituent of gephyrean muscle, in which it takes the place of arginine phosphate. This conclusion harmonizes with the belief that there are close affinities between the Gephyrea and the Annelida, since many of the latter also contain 'AP'.

The position of the Oligochaeta and the nature of their phosphagen, if indeed they contain any phosphagen, remains for further exploration and we hope also to have opportunities to study the other annelid classes. In the meantime it is only possible to conclude that the Annelida, including the Gephyrea to which, it is generally held, they are closely related, are probably unique among the major invertebrate phyla in their possession of a new and so far unidentified arginine phosphate-like compound, 'AP'. The Annelida are remarkable also in that numerous species contain this new substance side by side with a second phosphagen which may be creatine phosphate, while in some species this creatine phosphate-like compound only is present. These facts are the more striking when it is remembered that the Mollusca and Arthropoda, whose community of ancestry with the Annelida is generally recognized, have yielded abundant evidence of containing arginine and arginine phosphate but no trace of any creatine phosphate-like phosphagen (Kutscher & Ackermann 1926, 1933, 1936; Baldwin 1933*a*).

#### NOTE ON PHOSPHAGEN IN ECHINODERMATA AND PROTOCHORDATA

Within 20 years of its discovery, creatine had been found as a constituent of the muscles of representative members of all the classes of true Chordata and even in the cephalochordate *Amphioxus* (Hunter 1928). The extensive researches of Kutscher and his collaborators and others (reviewed by Kutscher & Ackermann 1926, 1933, 1936) led to the conclusion, strongly upheld by Hunter (1928), that creatine is characteristic of the muscles of vertebrates and replaced in those of invertebrates by arginine. Meyerhof (1930, p. 93) regarded this change-over from arginine to creatine as 'a characteristic, chemical mutation', a conclusion which, in the main, appeared to be upheld by the comparative studies of phosphagen distribution carried out by Eggleton & Eggleton (1928), Meyerhof (1928), Needham

*et al.* (1932) and others who have interested themselves in the problem. But the work of the latter group, in which particular attention was paid to the Echinodermata and Hemichordata, led to the discovery of certain exceptions to the general rule. This discovery has considerable bearing upon the problem of the origin of the vertebrates and it has seemed to us desirable here to review the position in the light of more recent work and a number of new observations (see table 3).

In the work of Needham *et al.* (1932), a substance having the properties of arginine phosphate was found in an asteroid, *Asterias glacialis* (= *Marthasterias glacialis*), from which arginine itself has been isolated by Ackermann (1935). The presence of arginine in three other asteroid species, *Pisaster ochraceus*, *Patiria miniata* and *Pycnopodia helianthoides*, was demonstrated by Arnold & Luck (1933) by the arginase-xanthidrol method, so that it seems reasonable to suppose that arginine phosphate is a general constituent of asteroid muscle. Creatine phosphate-like substances have not been detected.

Among Holothuroidea, arginine phosphate was found in *Stichopus* sp. and *Holothuria tubulosa* by Meyerhof (1928), who showed that the free base is attacked by arginase with production of urea, and a similar substance was demonstrated in *Synapta inhaerens* (= *Leptosynapta inhaerens*) by Needham *et al.* (1932). Baldwin & Needham (1937) later demonstrated the enzymic synthesis from added arginine of arginine phosphate by muscle extracts prepared from *Holothuria tubulosa* a species from which arginine itself was isolated by Kutscher & Ackermann (1931). Arnold & Luck (1933) further demonstrated the presence of arginine in *Stichopus californicus* and we ourselves have found arginine phosphate in *Thyone briareus*. Creatine and creatine phosphate have not been detected: the claim of Verjbinskaya, Borsuk & Kreps (1935) that *Cucumaria frondosa* contains both arginine phosphate and creatine phosphate has found neither confirmation nor parallel among the Holothuroidea. A careful and detailed examination of their papers has failed to convince us that the data presented support this claim. It is therefore probably not significant in this connexion that Needham *et al.* (1932) found no phosphagen at all in two specimens of muscle from *C. planci* but it is noteworthy that Haurowitz & Waelsch (1926) were unable to detect either creatine or creatinine in extracts of *Holothuria tubulosa*. It is, of course, conceivable that there may be species-specific differences among holothuroids just as there are in certain echinoids (see below) but, all in all, it is probable that the muscles of the holothurians contain arginine phosphate but not the creatine analogue, at any rate as a general rule.

The class Crinoidea has been but little studied. A substance behaving like arginine phosphate was demonstrated in *Antedon mediterranea* by P. M. Baldwin (cited in Baldwin & Needham 1937) and similar results were obtained with specimens of *A. bifida* by one of us (W.H.Y.) at Plymouth.

In the arms and disks of *Ophioderma longicauda* (Ophiuroidea) arginine phosphate is not present (Baldwin & Needham 1937) and the same is true of *O. brevispina* and *Ophiothrix fragilis* (present work). In both cases a substance with the properties of creatine phosphate was found and the presence of apparent creatine phosphate without arginine phosphate seems, among the Echinodermata, to be uniquely confined to the Ophiuroidea.

TABLE 3. DISTRIBUTION OF PHOSPHAGENS IN ECHINODERMS AND HEMICHORDATES

	mg. P/100 g.					Walpole test	Sakaguchi test	reference
	inorganic phosphate	creatine phosphate	arginine phosphate	total phosphagen	total			
ECHINODERMATA								
Crinoidea								
<i>Antedon bifida</i> (whole)*	2.2	0.0	2.6	4.8	2.6	o	o	
<i>A. mediterranea</i> (whole)	—	0.0	trace	—	trace	o	o	b
Asteroidea								
<i>Marthasterias glacialis</i> (tube feet)	1.9	0.0	6.1	8.0	6.1	o	o	a
Holothuroidea								
<i>Cucumaria planci</i> (body wall muscles)	7.7	0.0	0.0	7.7	0.0	o	o	a
<i>Synapta inhacrens</i> (body wall)	35.0	0.0	12.2	47.2	12.2	o	o	a
<i>Thyone briareus</i> (body wall muscles)	3.9	0.0	7.0	10.9	7.0	o	o	
<i>Th. briareus</i> (body wall muscles)	5.6	0.0	10.0	15.6	10.0	o	o	
<i>Th. briareus</i> (body wall muscles)	7.7	0.0	17.3	25.0	17.3	o	o	
Echinoidea								
<i>Arbacia punctulata</i> (jaw muscles)	15.0	0.0	15.8	30.8	15.8	—	+	
<i>Echinus esculentus</i> (jaw muscles)*	7.9	12.7	5.1	25.7	17.8	—	+	
<i>Strongylocentrotus lividus</i> (jaw muscles)	21.2	8.7	10.1	40.0	18.8	o	o	a
<i>S. lividus</i> (jaw muscles)	14.8	15.3	10.8	40.9	16.1	—	o	a
Ophiuroidea								
<i>Ophioderma brevispina</i> (arms)	3.0	3.6	0.0	6.6	3.6	+	o	
<i>O. brevispina</i> (arms)	6.5	3.4	0.0	9.9	3.4	—	—	
<i>O. brevispina</i> (disks)	9.8	3.1	0.0	12.9	3.1	—	—	
<i>O. longicauda</i> (arms)	—	7.4	0.0	—	7.4	o	o	b
<i>O. longicauda</i> (arms)	—	5.2	0.0	—	5.2	o	o	b
<i>O. longicauda</i> (arms)	—	5.6	0.0	—	5.5	o	o	b
<i>O. longicauda</i> (disks)	—	1.6	0.0	—	1.6	o	o	b
<i>Ophiothrix fragilis</i> (whole)*	10.0	5.5	0.0	15.5	5.5	—	—	
HEMICHORDATA								
<i>Balanoglossus salmoneus</i> (proboscis and collar)	16.0	6.0	6.0	28.0	12.0	o	o	a
<i>B. salmoneus</i> (region of gills)	22.5	3.9	0.0	26.4	3.9	o	o	a
<i>Saccoglossus kowalevskyi</i> (whole)	14.3	8.9	0.0	23.2	8.9	+	—	
<i>S. kowalevskyi</i> (whole)	10.0	6.3	0.0	16.3	6.3	+	—	

\* Experiments carried out at Plymouth.

o indicates that the Sakaguchi or Walpole test was not carried out.

References: (a) Data selected from Needham *et al.* (1932), (b) Data of P. M. Baldwin (cit. in Baldwin & Needham, 1937). All other data from present work.



Particularly interesting are the results that have been obtained with Echinoidea. According to Needham *et al.* (1932), the jaw-muscles of *Strongylocentrotus lividus* contain both arginine and creatine phosphates. This conclusion was supported by measurements of the velocity constants for acid hydrolysis of the compounds in presence and absence of molybdate ions, the values found agreeing well with those in the literature. Although the Vosges-Proskauer reaction was negative in extracts of these muscles, positive Jaffé reactions were obtained in autoclaved samples. Confirmatory evidence was provided by Baldwin & Needham (1937), who took advantage of the specificity of the phosphokinases responsible for the transference of phosphate from adenosine triphosphate to creatine and arginine respectively; Lehmann (1935) found that extracts from vertebrate muscles will not esterify arginine, nor will crab enzymes esterify creatine, and Ochoa (1938) similarly observed that fish muscle extracts will not esterify arginine nor lobster muscle extracts esterify creatine. Baldwin & Needham (1937) themselves found that extracts of the muscles of *Holothuria tubulosa* esterify arginine readily enough but have no action upon creatine. But, working with extracts prepared from the jaw muscles of *Paracentrotus lividus* and *Sphaerechinus granularis* they were able to demonstrate the specific enzymic synthesis, simultaneously or independently, of creatine phosphate from creatine and of arginine phosphate from arginine.

The identity of the arginine compound cannot be seriously doubted. The basic component is split by arginase with theoretical yields of urea (Baldwin & Needham 1937) and arginine itself has been isolated from *Arbacia pustulosa* by Holtz & Thielmann (1924). Arnold & Luck (1933) have also demonstrated the presence of arginine in *Strongylocentrotus franciscanus*.

Evidence for the identity of the creatine compound is less complete and has in fact been vigorously contested by Greenwald (1946). Greenwald believes that the apparent creatine phosphate found in Baldwin & Needham's experiments was an artefact due to reduction of the molybdate used in the colorimetric procedure by some unknown tissue constituent. If this objection were valid however, spurious creatine phosphate would have been expected to be found in every experiment in which this particular technique was employed, but, in point of fact, perfect blanks were obtained in all control experiments and in experiments where no creatine was added. Further, no creatine phosphate was found when creatine was supplied to extracts of the muscles of *Holothuria tubulosa* in experiments using the same procedures. Greenwald (1946) himself has isolated potassium creatinine picrate from the testes of two holothurians (*H. tubulosa* and *Cucumaria frondosa*) and of one echinoid (*Strongylocentrotus* sp.) and has produced evidence that creatine and conceivably even creatine phosphate may be present in the reproductive organs of a variety of invertebrates, but his experiments were not extended to cover the muscles, with which tissues our own work has been primarily concerned. We believe, therefore, that reasonable proof has been adduced for the presence of both arginine and creatine phosphates in the jaw muscle of the echinoids studied by Needham *et al.* (1932) and by Baldwin & Needham (1937). In more recent experiments on *Echinus esculentus* carried out at Plymouth, evidence was once more obtained by one of us (W.H.Y.) for the presence in the jaw muscle of two

phosphagens behaving with respect to molybdate like the arginine and creatine compounds.

The genus *Arbacia* seems however to differ from the other genera that have so far been studied. Holtz & Thielmann (1924), who isolated arginine from *A. pustulosa*, were unable to detect either creatine or creatinine in this species and we ourselves, working with trichloroacetic acid extracts of the jaw muscle of *A. punctulata*, found only arginine phosphate and no evidence whatsoever for the presence of creatine phosphate or of creatine itself.

Three different conditions thus exist among the Echinodermata. Arginine phosphate may be present alone (Crinoidea, Asteroidea, Holothuroidea) or may co-exist with creatine phosphate (Echinoidea). Finally, creatine phosphate may be present alone (Ophiuroidea). Clearly, therefore, the replacement of arginine by creatine is not to be regarded as a one-stage, mutation-like process but probably took place through an intermediate condition in which both compounds were present side by side.

The discovery that both phosphagens occur together in the muscles of certain echinoids is of interest from another point of view, for it lends chemical support to the morphological data upon which Bateson (1884-6) based his theory that the vertebrates evolved from an echinoderm stock by way of the Hemichordata. The chemical evidence gained further weight with the demonstration by Needham *et al.* (1932) that the tissues of the hemichordate *Balanoglossus salmoneus* contain two phosphagens, behaving like creatine phosphate and arginine phosphate respectively. Positive Vosges-Proskauer and Jaffé reactions served to confirm the presence of creatine phosphate but the evidence for the identity of the presumptive arginine compound rested solely upon its relative resistance to acid hydrolysis in the presence of molybdate ions. We hoped to obtain further confirmatory evidence during our stay at Woods Hole where another hemichordate, *Saccoglossus kowalevskyi*, was obtainable. In this species, however, only creatine phosphate was detectable and arginine phosphate was totally absent.\* Creatine phosphate was identified by its rapid hydrolysis in acid solutions in presence of molybdate and by positive Vosges-Proskauer reactions given in hydrolyzed extracts. This discrepancy between the two species would have been more surprising if we had not already found a similar discrepancy between *Arbacia* and the other echinoids previously studied.

#### GENERAL DISCUSSION: PHOSPHAGEN AND PHYLOGENY

It is generally accepted that radially symmetrical Coclenterata are widely separate from the rest of the Metazoa. Little is known of the phosphagen of the Cnidaria and Ctenophora. The scyphozoan, *Cyanea arctica* gave evidence of containing an arginine phosphate-like compound in the hands of Borsuk *et al.* (1933) while Mohr (1937) later isolated arginine itself from *C. capillata*. Needham *et al.*

\* Since this paper went to press we have had an opportunity of studying the recently discovered and closely related species, *Saccoglossus horsti*, through the kindness of Dr C. B. Goodhart. Like *S. kowalevskyi* this species was found to contain creatine phosphate but not its arginine analogue.

(1932) found no phosphagen in the tentacles of *Anthea rustica* and *A. cereus* (Actinozoa), though Arnold & Luck (1933) detected arginine in another actinozoan, *Metridium dianthus*. The only observation on ctenophores appears to be that of Needham *et al.* (1932), according to whom 42 % of the inorganic plus phosphagen P of *Pleurobrachia pileus* is accounted for by a substance behaving like arginine phosphate. Few though these data are they furnish indications that arginine phosphate may probably be regarded as the most primitive type of phosphagen at present known among the Metazoa.

There is no doubt that an arginine phosphate-like phosphagen occurs among Platyhelminthes according to the results of Needham *et al.* (1932), and the detection of arginine itself in *Leptoplana acticola* (Arnold & Luck 1933) seems to justify the conclusion that the primitive arginine phosphate is still present here.

The remainder of the Metazoa are broadly separable into two main groups. One of these groups, comprising phyla Mollusca, Arthropoda and Annelida, differs fundamentally in basic morphology and embryological development from the other, which comprises phyla Echinodermata and Chordata.

There is an abundance of evidence for the occurrence of arginine and arginine phosphate among Mollusca and Arthropoda (Kutscher & Ackermann, 1926, 1933, 1936; Baldwin, 1933*a*). However, as Arnold & Luck (1933) were the first to show, and as has been confirmed by our own observations, arginine phosphate seems to be characteristically absent from the Annelida and replaced there by some other phosphagen. Sometimes this new 'annelid phosphagen' is present alone, sometimes together with a second phosphagen which may well prove to be creatine phosphate. Sometimes also this second phosphagen is present alone, but nowhere among the Arthropoda or Mollusca is there any reason to believe that either the 'annelid phosphagen' or creatine phosphate is present.

Creatine phosphate has been found in members of the Echinodermata and Chordata but, in many cases among the echinoderms, arginine phosphate persists. The general conclusions reached in previous sections may be summarized conveniently in tabular form (table 4).

Within the echinoderm-chordate group, therefore, there is evidence of close relationships between echinoderms, hemichordates and vertebrates such as were

TABLE 4. DISTRIBUTION OF PHOSPHAGEN IN ECHINODERMATA AND CHORDATA

	arginine phosphate	creatine phosphate
ECHINODERMATA		
Crinoidea	+	—
Asteroidea	+	—
Holothuroidea	+	—
Echinoidea	+	+
Ophiuroidea	—	+
CHORDATA		
Protochordata		
Hemichordata	+	+
Cephalochordata	—	+
Vertebrata (all classes)	—	+

postulated on morphological grounds by Bateson (1884/6), who placed particular emphasis upon the fact that both the Hemichordata and the Echinodermata develop from larvae of the dipleurula type. Fell (1948), however, maintains that such larval resemblances are of minor importance compared to palaeontological data and writes: 'it is therefore obligatory to draw the conclusion that hemichordates do not exhibit any significant relationship with echinoderms.... The whole assumed relationship between the two phyla now rests upon some biochemical evidence of a slender character.' Although not qualified to criticize Fell's (1948) arguments on embryological or morphological grounds, we have nevertheless felt justified in reviewing here the biochemical evidence in the light of all the available data. It can be seen from table 4 that the chemical evidence does indeed support Bateson's postulate, although, admittedly, the nature of the phosphagen is merely one character and cannot, any more than any other single character such, for example, as metamerism, be regarded as the sole basis of, but only as supporting evidence for, phylogenetic relationships.

The replacement of arginine phosphate by creatine phosphate in the Ophiuroidea may perhaps reflect the same type of chemical specialization as occurred in the rise of the Chordata from ancestral echinoderms. In the Crinoidea, Asteroidea and Holothuroidea no replacement of the more primitive phosphagen has occurred except, perhaps, in a few species, while in some but not all Echinoidea it has been partly achieved.

Pending the positive identification of the phosphagens of the Annelida and Gephyrea it might be asked to what extent the discovery of two phosphagens side by side in certain members of this phylum affects earlier conclusions regarding the chemical support available for various theories of vertebrate evolution (cf. Needham & Needham 1932). Even if the creatine phosphate-like substance present in some annelids should prove to be identical with creatine phosphate itself it cannot, in our opinion, be held to indicate any close relationship between Annelida and Chordata such as was formerly postulated by Dohrn (1875) and Semper (1875, 1876). The Annelida, and with them the Gephyrea, have, unlike the Echinodermata, abandoned and replaced the primitive arginine phosphate and, if their second phosphagen is in fact creatine phosphate, the Annelida have apparently discovered and utilized this compound independently of the Echinodermata, presumably by convergent, chemical evolution.

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# Respiration of barley plants

## IV. Protein catabolism and the formation of amides in starving leaves

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In a continuation of earlier studies the nitrogenous constituents of barley leaves have been investigated with the object of gaining more direct evidence on the nature of protein catabolism in starving leaves. Particular attention was given to the origin and identification of the amides.

Tissue proteins were separated and partially analyzed by direct and indirect methods. The amino-acids of the barley-leaf protein were very similar to those of the proteins prepared from grasses. From these and other data estimates of the amide-N, glutamic acid and aspartic acid were obtained. The amide-N content of the whole protein of the leaves agreed closely with that of the separated protein. There was evidence that the release of amide-N proceeded uniformly as the proteins were broken down during starvation. Asparagine was identified as a product of catabolism by direct isolation from starved leaves; recovery of the crystalline product amounted to 60 % of the total stable amide-N of the crude extract. Glutamine was not isolated, but additional evidence of its presence in the leaves was obtained.

An analysis of the relation between proteolysis and formation of amides confirmed several of the suggestions made in a previous discussion. Most of the amide-N of the starved leaves was of secondary origin, and there were clear indications of a secondary synthesis of the aspartic acid combined in asparagine. Glutamine amide-N was formed secondarily during the early stages of starvation, but evidence of the origin of glutamic acid was inconclusive. The data of different experiments indicated that the breakdown of tissue proteins contributed between 20 and 40 % of the total carbon lost from the leaves as respiratory carbon dioxide.

### INTRODUCTION

The experiments described in part III of this investigation (Yemm 1937) show that amides play an important part in the metabolism of barley leaves. When the leaves are starved a large part of the tissue protein is rapidly converted to amides; the data indicate that extensive secondary changes of nitrogenous constituents may occur in close relation to the respiration of the leaves. However, it is well established that aspartic and glutamic acids are combined as amides in many plant proteins, and consequently asparagine and glutamine may be formed in living cells directly by proteolysis. This primary source of the amides must be taken into account in assessing their physiological significance in the metabolism of starving leaves. The results of the earlier experiments suggested that most of the unstable amide, probably glutamine, arises from the hydrolysis of the leaf protein, while the stable amide, mainly asparagine, may be formed by secondary changes more closely associated with respiration. In order to obtain more exact information on the relation between the amides and protein catabolism, the work has been extended in three general directions:

- (a) The separation and partial analysis of protein from barley leaves.
- (b) Determinations of the amide-N of the intact protein, and its changes during the course of starvation.
- (c) A more satisfactory identification of the amides of the leaves.

An account of these experiments is given in this paper, and their bearing on the problem of the metabolism of protein in the starving leaf is considered in a final section.

## I. PREPARATION AND ANALYSIS OF LEAF PROTEINS

It is chiefly owing to the researches of Chibnall and his collaborators, summarized by Chibnall (1939), that methods have been developed for the separation of proteins from green leaves. These methods have been followed as closely as possible in the present work, in order to allow a comparison of the results with the extensive data now available for other leaf proteins.

### 1.1. *Preparation*

The proteins were prepared from barley plants of the Plumage Archer variety, grown under normal field crop conditions during the season of 1936. Two harvests of leaves were made on 23 and 30 June, when the plants were 2 ft. 6 in. to 3 ft. high and the ears developing rapidly. The green leaves, usually 3 or 4 per shoot, were removed by hand, 4.5 kg. being obtained in the first collection and 5.0 kg. in the second.

On each occasion the protein was separated by the 'used ether water' method described by Chibnall, Millar, Hall & Westall (1933), the treatment being carried out as soon as possible after picking, so as to avoid loss of protein by hydrolysis. In samples of about 1 kg. the leaves were first washed with water, and then cytolized for 30 min. by means of 5 l. of ether water which had been used previously for the same purpose. The ether water was drained off and the leaves pressed to about 1 ton/sq.in. in a small hydraulic press and re-extracted twice with water, pressing as before. The residue was ground as finely as possible with a mincing machine, and then enclosed in a fine silk cloth for extraction with three successive quantities of 1 l. of distilled water. The dark green extract was filtered by suction through a well-rammed pad of filter-paper pulp on a large Buchner funnel. In this way a clear brown extract was obtained of 12 to 15 l. in the different experiments. Protein was separated from the extract by addition of 2N-HCl to give pH 4.5 to 4.7. After allowing the precipitate to settle, most of the liquid was siphoned off, and the protein, coagulated by standing in a boiling water-bath, was then separated by filtration, washed thoroughly with absolute alcohol and ether and dried at 100° C. In this way 29.5 g. of protein ( $E_1$ ) were prepared in the first experiment, and 41.2 g. in the second ( $E_2$ ). A small further precipitation of protein occurred in the first experiment on adding acid to the supernatant liquid removed by siphon. This protein was collected and dried as before yielding 3.5 g. ( $E_{1a}$ ); it was kept separate, so that it could be compared with the main preparation of the experiment.

After drying to constant weight at 105° C samples of each preparation were analyzed by the Kjeldahl method for total nitrogen. The replicate analyses agreed closely, the mean values, calculated as a percentage of the dry weight, for  $E_1$ ,  $E_{1a}$  and  $E_2$  all falling within the limits of  $13.9 \pm 0.1\%$ . The mineral ash content was uniformly low, having a mean value of 1.2% of the total dry weight, so that the nitrogen content of the ash-free protein would have been 14.1%.

Only a relatively small part of the protein of the leaves was separated in these experiments. Estimates of the total protein were made by indirect methods, which have been described already (Yemm 1937), and the results with calculated yields are set out in table 1.

TABLE 1. YIELD OF LEAF PROTEINS

preparations	wt. of leaves (kg.)	total protein (g. N)	separated protein (g. N)	% yield
E <sub>1</sub> , E <sub>1a</sub>	4.5	24.8	4.56	18.4
E <sub>2</sub>	5.0	27.5	5.68	20.6

Evidence has been assembled by Chibnall (1939) and Lugg (1939*b*) to show that, despite the relatively low yields, the proteins separated by the 'used ether water' method contain both cytoplasmic and chloroplastic components and are reasonably representative of the whole proteins of the leaf. Although nucleoproteins are probably not dispersed by the treatment, it is clear that this latter fraction represents only a small part of the total protein. The low yields, especially with fibrous leaves such as those of barley, are attributable to incomplete rupture of the cells during grinding rather than preferential extraction of different proteins. Some evidence that the present preparations are fairly representative, at least with regard to their content of amide-N, will be given in § 3.

### 1.2. Group analyses

Indirect group analyses of the hydrolyzed protein were made by the method of Van Slyke (1911), as modified by Damodaran (1931) to include an estimate of the dicarboxylic amino-acids. In this way the total-N of the hydrolysate was fractionated into the following groups: acid humin-N, alkali humin-N, amide-N, basic amino-N, dicarboxylic amino-N and monocarboxylic monoamino-N.

4.5 g. of the dry protein were hydrolyzed with 30 ml. of 20 % HCl (wt./vol.) by boiling under a reflux condenser for 24 hr. The acid humin was removed and thoroughly washed in the centrifuge, its nitrogen content being determined in the usual way. The main part of the hydrolysate was divided into three parts: in the first of these total-N and amide-N were determined in duplicate, total-N by the micro-Kjeldahl method, amide-N by distillation of the free ammonia *in vacuo* after the addition of borate buffer pH 9.5. The other two parts of the hydrolysate were used for duplicate determination of alkali humin-N, dicarboxylic amino-N and monocarboxylic monoamino-N, the experimental method following closely that described by Damodaran (1931). Experience with the method of precipitation of the calcium salts of the decarboxylic amino-acids was gained by the analysis of mixtures of pure aspartic and glutamic acids. In several experiments it was found that 95 to 98 % of the total-N of these acids could be recovered by precipitation of the calcium salts with 10 vol. of 95 % alcohol. The results of duplicate analyses of the protein preparations E<sub>1</sub> and E<sub>2</sub> are summarized in table 2.

A satisfactory recovery of total-N was made in each of the analyses, and there was no evidence of significant differences between the two preparations E<sub>1</sub> and E<sub>2</sub>.



With other leaf proteins Millar (1935) has shown that estimates of the basic amino-N made by the method of Van Slyke as above, were higher than those by the original method of Hausmann (1900), in which the phosphotungstates were separated in presence of sulphuric acid instead of hydrochloric acid. A direct comparison between the two methods was therefore made in the analysis of one of the preparations of the leaf protein of barley ( $E_1$ ). Duplicate estimates of basic amino-N by the Hausmann method, following the procedure of Millar, gave a value of  $21.6 \pm 0.3$  % of

TABLE 2. GROUP ANALYSES OF THE LEAF PROTEIN

	N as % of total N				
	prep. E <sub>1</sub>		prep. E <sub>2</sub>		mean
	(1)	(2)	(1)	(2)	
1. acid humin-N	2.3	2.3	2.2	2.0	2.2
2. alkali humin-N	2.1	2.2	2.0	2.4	2.2
3. amide-N	5.9	6.2	6.0	5.7	6.0
4. dicarboxylic amino-N	18.7	18.1	18.9	18.5	18.6
5. basic amino-N	26.2	25.8	26.6	26.2	26.2
6. monocarboxylic monoamino-N	44.1	44.3	42.7	44.1	43.8
7. total-N of fractions	99.3	98.9	98.4	98.9	98.9

the total-N, compared with  $25.9 \pm 0.4$  % by the Van Slyke method. Millar (1935) suggested, on the basis of a comparison with direct methods of isolation, that the Hausmann value was the more accurate estimate of basic amino-acids of the protein; but it is possible that the solubility of arginine phosphotungstate under the conditions of the separation is mainly responsible for the discrepancy between the two indirect methods (Tristram 1939). It seems clear that estimates of total basic amino-acids, made by either of the above procedures, are of comparative rather than absolute value.

### 1.3. *Estimation of amide-N*

The amide-N of a protein, determined after prolonged hydrolysis with relatively strong acid, as described in the previous section, is usually too high. Gortner & Holm (1919) have shown that a continuous release of ammonia occurs under these conditions owing to slow decomposition, which is now known to involve mainly the hydroxy-amino-acids serine and threonine (Rees 1946). Owing to the special importance of the amide fraction of the leaf protein in the present research, a more accurate estimate was sought, both by following the course of ammonia production during hydrolysis, as suggested by Shore, Wilson & Stueck (1936), and by hydrolysis with more dilute acid.

Weighed samples of 0.2 to 0.3 g. of the dried and powdered protein were mixed with 10 ml. of 20 or 5 % (wt./vol.) HCl. The mixture was heated under a reflux condenser in a boiling water-bath for a known time, varying in the different experiments from 15 min. to 10 hr. The release of ammonia was followed by transferring the partially neutralized hydrolysate to the apparatus of Parnas & Heller (1924), addition of borate buffer pH 9.5 and distillation into standard acid for 20 min. at a pressure of 1 to 2 cm. of mercury. Comparative tests with the different protein

preparations were in good agreement, and the results obtained with one of them ( $E_2$ ) are summarized graphically in figure 1.

There is little doubt that the rapid release of ammonia during the first few hours represented the hydrolysis of amide linkages in the protein, while the subsequent slow increase was owing to the secondary decomposition of unstable amino-acids. Assuming that the secondary decomposition goes on continuously during hydrolysis, a correction can be made by extrapolation (broken line in figure 1) as indicated by Shore *et al.* (1936). The secondary production of ammonia is very much slower with dilute acid, but after correction the true amide figures agree closely (4.70 and 4.65 %). This is regarded as a much more accurate estimate of amide-N than the mean value of 6.0 % recorded in the previous section.

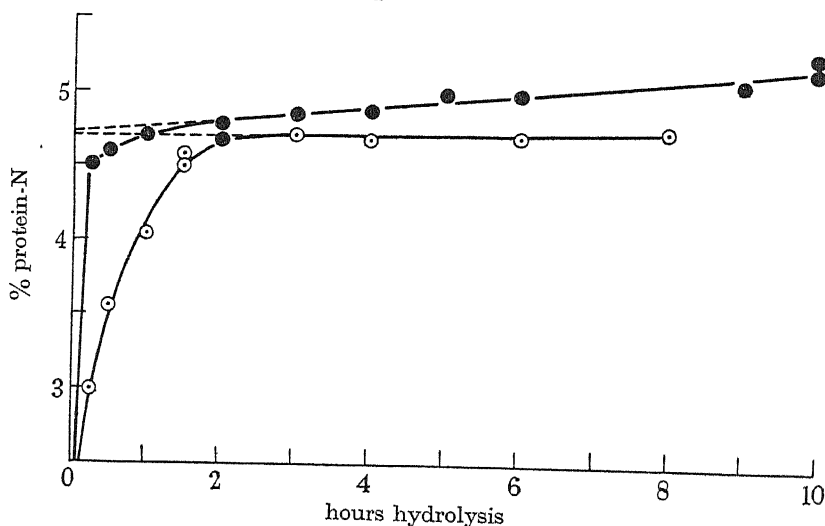


Figure 1. The release of ammonia during hydrolysis of leaf protein (preparation  $E_2$ ) at 100° C with 5 % (wt./vol.) hydrochloric acid ○ and with 20 % (wt./vol.) hydrochloric acid ●. The values estimated by extrapolation were 4.65 and 4.7 % of the total-N of the protein.

#### 1.4. Separation of glutamic and aspartic acids

The separation of the dicarboxylic amino-acids was effected by the method of Foreman, as modified by Jones & Moeller (1928) and Millar (1936). More recently these methods have been examined critically by Bailey, Chibnall, Rees & Williams (1943); it is clear that the procedure adopted here does not give a complete separation, the recovery of aspartic acid being more seriously affected. A brief description of the results is given below, since they may be compared directly with the analyses of grass proteins carried out by Millar, who used a similar method.

35 g. of the dried protein were heated with 200 ml. of 20 % HCl on a boiling water-bath for 2 hr. and then boiled gently under a reflux condenser for 30 hr. in an oil bath. Acid humin was removed by filtration and thoroughly washed with hot dilute acid; dried to constant weight at 105° C the humin weighed 2.55 g. and contained 0.080 g. of nitrogen. The filtrate contained 4.79 g. of nitrogen, making a total of 13.9 % of the dry weight of the protein. Most of the hydrochloric acid was removed by evaporation *in vacuo* to a syrup, and the solution was made alkaline

to litmus by the addition of recrystallized barium hydroxide. The precipitated alkali humin was removed by filtration; after drying it weighed 3.59 g. and contained 0.081 g. of nitrogen.

The filtrate, freed from humin, was saturated with barium hydroxide and slowly poured into 5 vol. of 90 % alcohol with vigorous stirring. After standing for 48 hr. at room temperature the precipitate was filtered off on a small Buchner funnel, washed with absolute alcohol and most of the alcohol removed by drying for a short time *in vacuo* at 30° C. The barium salts were dissolved in 500 ml. water, and a small amount of insoluble matter removed by the centrifuge and washed three times with warm water; the resultant solution contained 1.27 g. of nitrogen or 26 % of the total protein-N. After resaturation with barium hydroxide, the dicarboxylic acids were again precipitated by addition to 1.3 vol. of 90 % alcohol and standing on ice for 24 hr. This precipitate was collected by filtration, washed with alcohol as before, and reserved for further treatment. The alcoholic filtrate was evaporated to dryness *in vacuo* with the air inlet protected by a soda-lime tube, the residue being dissolved in 160 ml. of warm water and a second precipitate of barium salts made as before. A third precipitation from 60 ml. of water was carried out in a similar manner. The combined precipitate collected from the three successive treatments was taken up in water, and decomposed by addition of a slight excess of sulphuric acid. Barium sulphate was removed in the centrifuge and washed thoroughly three times with hot water. Sulphuric acid was finally removed exactly as barium sulphate. The solution of the dicarboxylic acids separated in this way contained 0.98 g. of nitrogen or 20.1 % of the total-N of the protein.

*Glutamic acid* was isolated as hydrochloride by evaporating the solution *in vacuo* to 50 or 60 ml. and then saturating it with dry hydrochloric acid gas at 0° C. After standing on ice for 1 week, the crystalline deposit was collected on sintered glass, washed with a little cold strong hydrochloric acid, and dried *in vacuo* over potash and calcium chloride. In this way 2.44 g. of the hydrochloride was obtained. The mother liquor and washings were concentrated to about 30 ml. and a second crop of crystals removed (1.12 g.), a final crop of 0.87 g. being obtained after evaporation to 15 or 20 ml. and standing for 2 weeks on ice. The total yield was 4.43 g., and after recrystallizing once it had a nitrogen content of 7.54 % ( $C_5H_9O_4N.Cl$  required 7.63 %). Correcting for samples withdrawn for analysis, it represented 3.7 g. of free glutamic acid or 10.6 % of the total dry weight and 7.1 % of the total-N of the protein.

*Aspartic acid* was separated as the copper salt from the mother liquor after the removal of glutamic acid hydrochloride described above. The solution, containing 0.62 g. of nitrogen, was evaporated *in vacuo* and taken up in 100 ml. of water. The solution was saturated with barium hydroxide, the barium salts being precipitated by 1.5 vol. of alcohol and standing on ice for 24 hr. The precipitate, separated by filtration, was decomposed and freed from sulphate as before, yielding a solution containing 0.27 g. of nitrogen. After evaporation *in vacuo* to 150 ml. the solution was boiled for 15 min. with an excess of freshly prepared and thoroughly washed cupric hydroxide, and then filtered through a steam-heated funnel. In two successive crops 3.02 g. of air-dried copper salt was collected. After recrystallizing once it

contained 5.17 % N ( $C_4H_5O_4N \cdot 4\frac{1}{2}H_2O$  requires 5.09 % N). Corrected for the samples withdrawn for analysis, this represented 1.62 g. of free aspartic acid, or 4.6 % of the weight and 3.5 % of the total-N of the protein preparation.

### 1.5. Comparison with other leaf proteins

With regard to their constituent amino-acids, the most fully investigated leaf proteins are those separated from forage grasses, such as *Dactylis glomerata* (Chibnall, 1939). It is of interest, therefore, to compare the present results with analyses of the cocksfoot grass proteins which have been made by similar methods; the available data are summarized in table 3.

TABLE 3. COMPARISON OF BARLEY AND COCKSFOOT GRASS PROTEINS

	% protein-N*	
	barley	cocksfoot grass
1. amide-N (20 % HCl, 24 hr.)	6.0 ± 0.3	6.1 ± 0.2 <sup>(1, 2)</sup>
2. amide-N (5 % HCl, 3 hrs.)	4.8 ± 0.1	5.1 ± 0.1 <sup>(1, 5)</sup>
3. basic amino-N (Hausmann)	21.6 ± 0.3	21.6 ± 1.6 <sup>(2)</sup>
4. basic amino-N (Van Slyke)	25.9 ± 0.4	28.7 ± 1.1 <sup>(1, 2)</sup>
5. dicarboxylic amino-N	18.6 ± 0.5	19.9 <sup>(1)</sup>
6. glutamic acid-N	7.1	8.03 <sup>(3)</sup>
7. aspartic acid-N	3.5	3.25 <sup>(3)</sup>
8. humin-N	4.4 ± 0.2	5.9 ± 0.6 <sup>(1, 3)</sup>
9. total-N (as % ash-free dry matter)	14.1 ± 0.1	13.4 ± 0.6 <sup>(1, 4, 5)</sup>
(1) Millar & Chibnall (1932). (2) Millar (1935). (3) Millar (1936).		
(4) Lugg (1939b). (5) Chibnall (1939).		

\* Where a number of estimates on the same or different preparations of the protein are available, the mean has been calculated and the maximum variation is shown alongside.

A close similarity is apparent in all the groups into which the total-N has been fractionated. Despite the uncertainty of the indirect methods of analysis, there is little doubt that the leaf proteins of these related species resemble one another closely with regard to their constituent amino-acids. From the results collected together by Chibnall (1939), it is apparent that the similarity extends to other species of the Gramineae and to a more limited degree to species of the Leguminosae and Chenopodiaceae. As discussed by Lugg (1939b, 1940), real differences of composition probably exist, at least between different families, although they may be in part attributable to different proportions of cytoplasmic and chloroplastic proteins in the preparations. It is an important deduction from these observations that the metabolism of protein in different leaves may be associated with a much more uniform assembly of amino-acids than might at first be suspected.

The estimates of aspartic and glutamic acids of the cocksfoot grass protein, shown in table 3, are those recorded by Millar (1936), who used a somewhat different method for the separation of aspartic acid. In the light of more recent analyses by improved methods, Chibnall (1939) has withdrawn these estimates of the dicarboxylic amino-acids; more accurate values are, glutamic acid 8.0 % and aspartic acid 4.9 % of the total protein-N. From the evident similarity of the two proteins, it is considered that these latter values are the best estimates as yet available for the

dicarboxylic amino-acids of the barley-leaf protein. The determinations of total dicarboxylic amino-N made by the method of Damodaran (1931) are clearly excessive, and there is now decisive evidence that the Foreman precipitation does not give a specific separation of aspartic and glutamic acids. As already noted in the case of the basic amino-N fractions, these indirect group analyses are of comparative rather than absolute value.

Nearly all of the proteins separated from leaves by the 'used ether water' method have a relatively low nitrogen content. There is evidence from the formation of humin during acid hydrolysis that the preparations are contaminated by mucilages or pentosans (Millar 1936; Lugg 1939*a*). The analyses of the barley-leaf protein are open to a similar interpretation. As shown in §1.4, the small amount of nitrogen separated with the humin is associated with a substantial proportion of the dry weight of the protein; 35 g. of the preparation containing 4.87 g. of nitrogen yielded 5.94 g. of humin containing 0.16 g. N. It may be calculated, therefore, that the protein, if freed from contaminant and ash, would have weighed 29.5 g. and contained 4.71 to 4.87 g. of nitrogen, that is, 15.9 to 16.2 %, a value agreeing more closely with that of purer plant proteins. The chemical nature of the contaminant is at present uncertain, but it is very probably intimately associated with the protoplasmic proteins, possibly as an intermeshed colloidal system (MacDougal 1920). In relation to the present research it is of interest that the breakdown of cell proteins may be associated with a liberation of these complex carbohydrates. Some evidence has already been obtained of the accumulation of non-fermentable carbohydrates during protein catabolism in starved leaves (Yemm 1935).

## 2. AMIDE NITROGEN OF THE LEAF PROTEIN AND ITS CHANGES DURING STARVATION

The preparations, made from the leaves by the 'used ether water' method, are in all probability complex mixtures of the physiologically active proteins, derived from the cytoplasm and chloroplasts. When the cell structures break down in starved tissues, it is possible that the various components of the proteins are attacked at different rates. Wood, Cruickshank & Kuchel (1943) have obtained evidence that a differential rate of loss of chloroplastic and cytoplasmic protein occurs in the starved leaves of some grasses. These considerations must be borne in mind when applying the results of analysis of the preparations to the problem of protein metabolism in the leaves. The rapid accumulation of soluble amides at certain phases of starvation might be accounted for by the hydrolysis of a structural protein having a high content of amide-N. An attempt has been made, therefore, to estimate the amide-N of the whole protein of the leaves and the changes which take place when the tissues are progressively starved. In this way it is possible to gain more direct data on the relation between proteolysis and the formation of soluble amides in the leaves. There is strong evidence that nearly all the insoluble N of leaves is combined in the form of protein, and determinations of amide-N were made on the insoluble residue, from which the soluble constituents had been exhaustively extracted by the method already described (Yemm 1937). The residue was dried at 80° C and powdered, weighed samples being used for micro-Kjeldahl

analysis of total-N, and for hydrolysis with 5 % (wt./vol.) HCl followed by distillation of the ammonia *in vacuo*, as described in §2.3. Thus the amide-N could be calculated as a percentage of the total insoluble N of the tissue.

The results of a test with freshly detached leaves, in which different periods of hydrolysis were used, are summarized in figure 2, together with similar data obtained with the protein preparation ( $E_2$ ) for comparison. The apparent amide-N of the whole protein was higher than that of the separated protein, but it was evidently associated with a much more rapid release of ammonia by secondary breakdown during the course of hydrolysis. After correcting by extrapolation as before, a value of 4.70 was obtained for the whole tissue protein, as against 4.65 % for the preparation. The agreement between these estimates gave direct evidence that the separated protein was reasonably typical of the whole tissue proteins at least with respect to its amide-N.

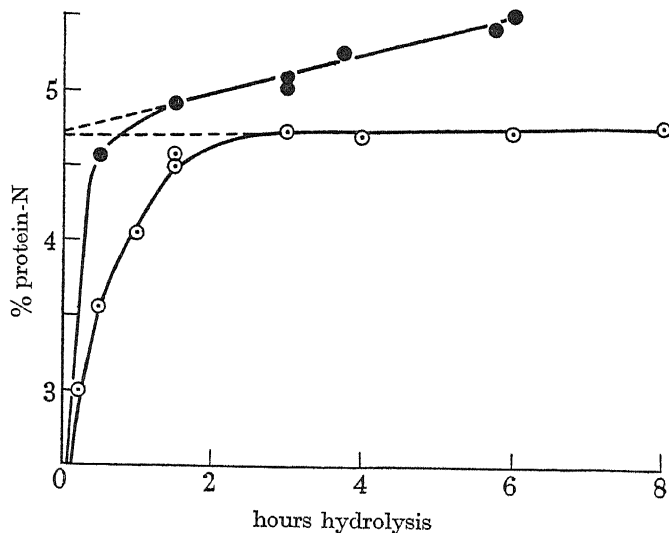


Figure 2. The release of ammonia during hydrolysis with 5 % (wt./vol.) hydrochloric acid at 100° C of insoluble leaf residue ● and protein preparation  $E_2$  ○. Amide values estimated by extrapolation were 4.7 and 4.65 % of the total-N of the protein.

A period of 3 hr. hydrolysis with 5 % acid was adopted for routine analyses of the insoluble residues of fresh and highly starved leaves. Although the estimates made in this way are somewhat high, the errors introduced are not serious for comparative purposes. The changes in amide-N of the tissue protein in starving leaves were investigated in three experiments during 1937 and 1939; a typical series of results is shown in table 4.

During starvation over 80 % of the protein initially present in the leaves was broken down, but the amide-N, as shown in column 5 of the table, was always within the limits 4.8 to 5.3 % of the total insoluble nitrogen. Similar observations were made in the other two experiments; in each case there was a tendency for the higher values of amide-N to be obtained with highly starved leaves. The errors of analysis are inevitably increased in these samples of low nitrogen content, and there is the possibility of interference from other non-protein constituents, so that

no great weight can at present be placed on this apparent increase in the proportion of amide-N.

In general, the results show that no preferential breakdown of structural proteins rich in amide-N could account for rapid accumulation of soluble amides at any phase of starvation. Either the different components of the cell proteins are very similar with regard to their content of amide-N, or they are broken down in the

TABLE 4. CHANGES OF PROTEIN-N AND PROTEIN AMIDE-N IN STARVING LEAVES

sample no.	hours starved	mg. N/1 g. fr. wt.		amide % protein N
		total insoluble N	amide-N	
1	0	7.26	0.351	4.8
2	12	6.98	0.340	4.9
3	24	5.74	0.286	5.0
4	36	4.86	0.240	4.9
5	48	4.05	0.193	4.9
6	60	3.06	0.160	5.2
7	72	2.48	0.132	5.3
8	84	2.01	0.106	5.2
9	96	1.64	0.080	4.9
10	108	1.60	0.085	5.3
11	132	1.42	0.075	5.3

starving leaves at about the same rate. In any case the liberation of amides by proteolysis seems to proceed uniformly; a reasonably accurate estimate of the amide-N of the whole tissue protein can be made as 4.7% of the total insoluble nitrogen of the residue, either with freshly detached or highly starved leaves. On the basis of this evidence it is possible to examine more fully the results of earlier experiments (Yemm 1937), in which the loss of protein and accumulation of soluble amides were measured.

### 3. THE SOLUBLE AMIDES OF STARVED LEAVES

Analyses of extracts from fresh and starved barley leaves by indirect methods have shown consistently that two amide fractions could be distinguished; some evidence for identifying them with asparagine and glutamine has already been discussed (Yemm 1937). With regard to glutamine, a valuable confirmation could be obtained owing to the distinctive reactions of this amide in the Van Slyke apparatus. No similar assurance could be gained in the case of asparagine. It was clear that other nitrogenous constituents, such as ureides, might contribute to the stable amide fraction, determined by the release of ammonia after hydrolysis with dilute acid. Particular attention was given, therefore, to the possibility of identifying asparagine by direct isolation from starved leaves.

#### 3.1. Isolation of asparagine

The leaves were harvested from a field crop of Plumage Archer barley on 6 July 1937. Their fresh weight was 3.7 kg. In the laboratory the leaves were loosely packed into a large metal container (2 ft. 6 in. × 1 ft. 6 in.) lined with damp muslin.

A rapid stream of air, saturated with water vapour, was distributed by six leads to the bottom of the container. The leaves were kept darkened by covering with black cloth and a loosely fitting lid. There was a rise of temperature in the chamber to 25 to 26° C at first, but it was maintained at 20 to 23° C for most of the starvation period. Yellowing of the tips of the leaves became appreciable after 40 hr., and followed a normal course. At suitable intervals samples consisting of fifty leaves were removed and analyzed in order to follow the accumulation of ammonia and amides in the tissues. The results are shown in table 5.

After 78 hr., when the stable amide had reached a high value, the leaves were treated for the extraction and separation of amides, following the methods described by Vickery, Pucher & Clark (1935). In samples of about 1 kg. they were first cytolyzed with ether and the sap collected at the press, the residue being washed twice with 750 ml. of distilled water. The combined extract was treated with a slight excess of basic lead acetate; the bulky precipitate, removed by filtration, was washed at the centrifuge with 2 l. of distilled water. An excess of mercuric nitrate reagent was then added, neutralized to litmus and the resultant precipitate collected and washed at the centrifuge. After decomposition by  $\text{H}_2\text{S}$ , mercuric sulphide was removed by filtration through paper pulp, and the solution, freed from  $\text{H}_2\text{S}$  by aeration, was neutralized with ammonia. It was then concentrated *in vacuo* to about 100 ml. At appropriate stages, samples of the extract were taken for estimation of the amide fractions; the results are summarized as follows:

	stable amide-N (g.)	unstable amide-N (g.)
(1) water extract	1.14	0.34
(2) after clearing with lead acetate	0.96	0.28
(3) decomposed mercuric precipitate	0.84	0.24

After addition of 1 vol. of 95 % alcohol to the concentrate and standing on ice, a crystalline deposit of 10.2 g. was removed in three successive crops. Analyses of the crystals and the mother liquor failed to show the presence of appreciable quantities of unstable amide. It seemed probable that the relatively small amount present in the extract was lost mainly during concentration, which with the apparatus available could not be carried out as rapidly as desirable. After recrystallizing twice from 25 % alcohol, a yield of 7.02 g. was obtained, which was identified as asparagine from its crystalline form and the following analytical data:

	found	calc. for $\text{C}_4\text{H}_8\text{O}_3\text{N}_2 \cdot \text{H}_2\text{O}$
total-N	18.4	18.67
amide-N (3 hr. $2\text{NH}_2\text{SO}_4$ )	9.27	9.33
amino-N (10 min. Van Slyke)	9.30	9.33
water of crystallization	12.09	12.00

Correcting for the samples withdrawn for analysis, the yield of asparagine represented 0.69 g. of stable amide-N, or 60 % of the stable amide-N of the water extract of the leaves and 82 % of the stable amide precipitated by mercuric nitrate. It is probable that a large part of the losses occurred in the removal of the bulky



precipitate produced by lead acetate, and in concentration of the extract, as noted above. Although the isolation of the smaller amount of glutamine was not achieved, it is noteworthy that the reactions of the unstable amide fraction during treatment with lead acetate and mercuric nitrate were fully consistent with the known properties of glutamine.

TABLE 5. ACCUMULATION OF AMMONIA AND AMIDES IN STARVED LEAVES

sample no.	hours starved	mg. N/10 g. fr. wt.		
		ammonia	unstable amide	stable amide
1	20	0.18	0.80	0.75
2	48	0.44	1.95	1.87
3	72	1.73	1.60	3.25

### 3.2. The hydrolysis of unstable amide in leaf extracts

Vickery and others (1935) have shown that glutamine may be hydrolyzed more selectively at pH 6.5 than at pH 4.0, as used at an earlier stage of this work. After 2 hr. at 100° C and at pH 6.5 the amide is converted quantitatively into pyrrolidone carboxylic acid and ammonia. Owing to the reaction of the amide group in the Van Slyke apparatus, the ratio of the loss of amino-N to increase of ammonia on hydrolysis should be close to 1.8. Determinations of this ratio were made on a series of extracts from starved barley leaves. The results are set out in table 6.

TABLE 6. CHANGES OF AMINO AND AMMONIA-N IN LEAF EXTRACTS  
HYDROLYZED AT pH 6.5 FOR 2 hr.

leaf sample no.	hours starved	mg. N/10 g. fr. wt.				
		ammonia		amino		(loss of amino-N)/ (gain in ammonia-N)
		initial	after hydrolysis	initial	after hydrolysis	
1	6	0.30	0.82	7.3	5.8	2.9
2	20	0.26	1.06	8.1	6.5	2.0
3	40	0.39	2.19	18.3	15.0	1.8
4	64	0.65	3.00	28.0	23.2	2.0
5	80	2.75	6.00	30.0	24.5	1.7
6	112	9.60	12.8	28.2	23.2	1.6

Within the limits of experimental error, the amino : ammonia ratios agree with those expected for glutamine, as already observed when the hydrolysis was carried out at pH 4.0 (Yemm 1937).

A second method, suggested by Vickery and others (1935), for the detection of interfering substances, depends on the fact that compounds, such as allantoin and urea, are only partially hydrolyzed to ammonia after 2 hr. at pH 6.5. Therefore, if appreciable amounts were present an increase of ammonia would occur when the heating period was extended to 4 hr. Comparative tests, in which the release of ammonia was measured after heating for 2 and 4 hr., are summarized in table 7.

TABLE 7. RELEASE OF AMMONIA AFTER HYDROLYSIS FOR 2 AND 4 HR. AT pH 6.5

sample no.	hours starvation	mg. N/10 g. fr. wt.			
		amide-N pH 6.5-	amide-N pH 6.5-	$\Delta$	asparagine correction
		2 hr.	4 hr.	(4)-(3)	(0.026 $\times$ stable amide)
(1)	(2)	(3)	(4)	(5)	(6)
1	6	0.52	0.52	0	0.01
2	20	0.80	0.85	0.05	0.02
3	40	1.80	1.95	0.15	0.09
4	64	2.35	2.50	0.15	0.21
5	80	3.25	3.45	0.20	0.30
6	112	3.20	3.35	0.15	0.25

A small but appreciable increase of ammonia (col. 5) was generally found after more prolonged hydrolysis, varying from 0 to 8.3% of the glutamine amide-N. Asparagine was present in considerable amounts, especially in the more highly starved samples no. 4, 5, 6 and there is evidence that the increase of ammonia was mainly owing to the slow hydrolysis of the stable amide. Vickery and others (1935) have shown that about 2.6% of asparagine amide-N is hydrolyzed in 2 hr. under these conditions. A correction, calculated in this way from the determination of stable amide-N in the different extracts, is given in column 6, table 7. It is apparent that asparagine accounts for the increase of ammonia, within the limits of experimental error, and therefore seems to be the only substance interfering appreciably with the determination of glutamine in extracts prepared from starved leaves.

#### 4. THE ORIGIN OF AMIDES IN STARVED LEAVES

In this section the data will be considered in so far as they throw further light on the course of protein catabolism and formation of amides in starving leaves. With regard to the identity of the soluble amides, the evidence given in § 3 is now sufficiently strong to justify the conclusion that asparagine and glutamine are the main constituents of those fractions which have been described hitherto as stable and unstable amides. The possibility that other nitrogenous constituents make a small contribution to the indirect determinations cannot be ruled out, but it is very unlikely that this could seriously influence the preliminary analysis of the results which will be undertaken here.

An estimate of the extent to which the amides may be formed directly by proteolysis can now be made. From the evidence already given, the best estimates of the amide-N and dicarboxylic amino-acids in the tissue proteins are summarized as follows:

	% protein-N
(1) amide-N	4.7
(2) glutamic acid-N	8.0
(3) aspartic acid-N	4.9

The amounts of either glutamic or aspartic acids are sufficient to combine with the whole of the amide-N, and there is at present no means of deciding whether the amide is held in asparagyl or glutamyl linkages of the protein. Nevertheless, it is

possible to estimate the *maximum* amount of either amide which could arise from proteolysis by assuming that the amide-N is linked exclusively with one or other of these amino-acids. Thus, if the amide group were linked entirely with glutamic acid, a maximum of 9.4% of the protein-N could be liberated as glutamine, while a similar amount of asparagine might arise on the alternative assumption. Experiments, in which the breakdown of tissue proteins and formation of soluble amides have been measured, will be re-examined on the above basis.

It is appropriate first to consider the origin of the total amide-N which accumulates in starved leaves. Under the controlled conditions, which have been used in these experiments, the soluble amides rise to a peak value after about 100 to 130 hr. starvation, at a time when the leaves have yellowed and a substantial part of the tissue protein has been broken down. Since the protein contains 4.7% of its nitrogen in amide linkages, the amount of amide-N which could have been derived from proteolysis can be calculated. The results of nine experiments carried out between 1934 and 1938 are summarized in table 8. Details of some of these experiments have been described in part III of this series.

TABLE 8. ORIGIN OF THE AMIDE-N IN STARVED LEAVES

		mg. N/g. fr. wt.				
	experiment no.	hours starved	loss of protein		soluble amide-N formed	secondary amide-N
			total N	amide-N		
1	VI (1934)	114	5.76	0.27	1.90	1.63
2	VII (1934)	108	4.80	0.23	1.44	1.21
3	IX (1935)	96	3.31	0.16	0.95	0.79
4	X (1935)	88	2.59	0.12	1.11	0.99
5	XI (1935)	96	2.72	0.13	1.01	0.88
6	VII (1936)	88	4.68	0.22	1.32	1.10
7	VIII (1937)	144	5.84	0.27	1.64	1.37
8	XIII (1938)	120	5.05	0.24	1.45	1.21
9	XIV (1938)	122	6.03	0.28	1.86	1.58

The results of all the experiments show that by far the largest part of the soluble amides must have arisen by secondary changes; proteolysis could account for only 10 to 20% of the total amide-N. Coupled with the fact that ammonia generally accumulates in the yellowing leaves, it is clear that secondary breakdown of protein is highly active in the starved tissues, and may make an important contribution to their respiration.

Quantitatively, asparagine is usually the more conspicuous amide. Many experiments have shown that it is formed rapidly from about 24 to 120 hr., and the relation between this amide and the liberation of aspartic acid and amide-N from tissue protein, is analyzed in table 9. The calculated amounts of aspartic acid and amide-N, which could have arisen by proteolysis, are shown alongside the estimates of asparagine formed in the leaves over the corresponding period of starvation. It is evident that neither the aspartic acid nor the amide-N of the tissue protein is sufficient to account for the formation of the stable amide; the amount of asparagine present in starved leaves is generally 4 or 5 times greater than could be attributed

TABLE 9. FORMATION OF ASPARAGINE IN STARVED LEAVES

experiment no.	hours starved	mg. N/1 g. fr. wt.				
		asparagine formed (2 × stable amide)	loss of protein			initial amino-N
			total-N	aspartic acid	amide	
VI (1934)	114	1.88	5.76	0.28	0.27	0.93
VII (1943)	108	1.80	4.80	0.24	0.23	0.69
IX (1935)	96	1.56	3.31	0.16	0.16	1.00
X (1935)	88	1.42	2.59	0.13	0.12	0.37
XI (1935)	96	1.28	2.72	0.13	0.13	0.56
VII (1936)	88	2.08	4.68	0.23	0.22	0.56
VIII (1937)	144	2.74	5.84	0.29	0.27	0.41
XIII (1938)	120	2.58	5.05	0.25	0.24	0.20
XIV (1938)	122	2.86	6.03	0.30	0.28	0.38

directly to protein hydrolysis. Aspartic acid may be present in appreciable amounts in the free amino-acids of freshly detached leaves, and estimates of the total amino-acid have therefore been shown in table 9. In several of the experiments (X, VII (1936), VIII, XIII) it is clear that even if the whole of free amino-N initially present were aspartic acid, it would be inadequate to meet the requirements for the synthesis of asparagine. These data, therefore, provide strong evidence that both the aspartic acid and the amide-N of asparagine are formed secondarily in the starved tissues.

Glutamine, in contrast to asparagine, generally accumulates most rapidly during the early phases of starvation, when the leaves still contain relatively large amounts of carbohydrate (Yemm 1935, 1937). Accordingly, the relation between this amide and proteolysis has been examined over the first phase of starvation, 18 to 30 hr. The results are summarized in table 10.

TABLE 10. FORMATION OF GLUTAMINE IN STARVED LEAVES

experiment no.	hours starvation	mg. N/g. fr. wt.			
		glutamine (2 × unstable amide)	loss of protein		
			total-N	amide	glutamic acid
VI (1934)	30	0.26	1.12	0.053	0.089
VII (1934)	18	0.54	0.90	0.042	0.072
IX (1935)	24	0.42	0.94	0.046	0.075
X (1935)	20	0.49	1.10	0.052	0.088
XI (1935)	24	0.53	1.21	0.057	0.097
VII (1936)	24	0.07	0.79	0.037	0.063
VIII (1937)	24	0.12	1.27	0.060	0.102
XIII (1938)	24	0.12	1.05	0.049	0.084
XIV (1938)	24	0.22	1.68	0.079	0.134
XV (1938)	24	0.32	1.34	0.063	0.107

There is some variation in the results of the different experiments. In most of them the amount of glutamine amide-N formed in the leaves far exceeds that which could originate from the breakdown of tissue protein alone. The difference reaches an extreme in experiment VII (1934), when the increase of unstable amide after

18 hr. starvation was over six times greater than the estimated loss by proteolysis. On the other hand, in experiment VII (1936), VIII (1937), the breakdown of protein was adequate to provide the whole of the glutamine. It must be kept in mind, however, that the calculated values rest on the assumption that the whole of the amide-N of the protein is liberated as glutamine, so that the secondary formation of the amide in these latter experiments is not excluded. Data presented elsewhere indicate that the variation in the different experiments is attributable mainly to the age of the leaves and their sugar content at the time of detachment from the plants. Similarly, glutamic acid is not usually released from tissue proteins in sufficient quantity to meet the requirements of amide synthesis, but here the free amino-acids of the leaves must be taken into account. The amino-N, present in the freshly detached leaves, is shown in table 9, and could in all the experiments provide enough glutamic acid for the formation of glutamine. A further investigation of the free amino-acids is necessary before it can be decided whether glutamic acid, as well as the amide-N, is formed by secondary changes in the early stages of starvation.

Glutamine does not usually accumulate to such a great extent as asparagine, and the maximum amount is often reached at an earlier phase of starvation. An analysis of the changes associated with the maximum production of glutamine fully confirms that the amide-N must be mainly of secondary origin. But with regard to glutamic acid the evidence is again indecisive; it is possible that the free amino-acids of the leaves together with glutamic acid from proteolysis could meet in full the requirements for glutamine synthesis in most of the experiments. This interpretation does, however, often involve the assumption that practically the whole of the free amino-N of the leaves is in the form of glutamic acid.

Several of the views put forward in an earlier discussion of protein catabolism and respiration are here confirmed. Most of the amide-N and asparagine which accumulate in the starved leaves are of secondary origin, and their formation implies extensive breakdown of the products of proteolysis. It is necessary, however, to withdraw the suggestion that glutamine arises entirely from the hydrolysis of leaf proteins. There are clear indications that the formation of glutamine in the leaves is not a direct response to starvation conditions, but it is rather a part of the dynamic equilibrium in which the tissue proteins are maintained. The demonstration of the secondary formation of glutamine in leaves very soon after detachment from the plant suggests that the proteins may make a contribution to respiratory changes even at this early stage of starvation. Analysis of carbohydrates and measurements of the respiratory quotient indicate that most of the carbon dioxide arises at first by glycolysis (Yemm 1935); but it is possible that an exchange of carbon residues occurs between the products of glycolysis and the amino-acids, and this would not be readily detected by analytical methods. Gregory & Sen (1937) have already suggested that a continuous interchange of carbon residues of this nature is an important factor influencing the rate of respiration of barley leaves.

The amides, together with ammonia, make up a large part of the soluble nitrogenous compounds which are formed during protein catabolism. In most of the experiments between 60 and 70% of the total soluble nitrogen of highly starved

leaves can now be identified with reasonable certainty, as asparagine, glutamine and free ammonia. It is of interest, therefore, to make a provisional estimate of the overall contribution of proteins to the production of carbon dioxide in respiration. A balance sheet for carbon has been drawn up in table 11 to show the changes observed in eight of the experiments.

TABLE 11. CHANGES OF CARBON IN THE NITROGENOUS CONSTITUENTS OF STARVED LEAVES

experiment no.	hours starva- tion	mg. carbon/1 g. fr. wt.							lost as CO <sub>2</sub>
		loss of protein (N × 3.1)	gam of soluble N constituents					loss from protein	
			glutamine (N × 2.14)	aspara- gine (N × 1.71)	amino-N (N × 2.8)	other-N (N × 2)	total		
VI (1934)	144	18.0	3.59	3.21	3.67	1.70	12.2	5.8	23.5
VII (1934)	108	15.0	2.09	3.07	2.55	1.02	8.7	6.3	22.8
X (1935)	88	8.1	1.00	2.43	-1.03	-0.42	2.0	6.1	20.3
XI (1935)	96	8.5	1.56	2.18	-0.28	0	3.5	5.0	23.0
VII (1936)	88	14.6	1.18	3.56	1.43	1.16	7.3	7.3	19.5
VIII (1937)	144	18.3	1.15	4.68	2.57	2.00	10.4	7.9	22.9
XIII (1938)	120	15.8	0.68	4.41	0.67	3.68	9.4	6.4	25.9
XIV (1938)	122	18.8	1.58	4.82	1.82	1.56	9.8	9.0	22.2

The factors used for calculating the carbon content of the nitrogenous fractions are entered at the head of the columns. The chief uncertainty is in the amino-N and other-N fractions, where the factors are based on the average composition of amino-acids and plant bases respectively. Changes of these fractions during starvation are somewhat variable, and often relatively small, so that considerable fluctuations in their carbon content could not seriously affect the general result.

All the experiments show that a substantial loss of carbon from the nitrogenous constituents of the leaves is associated with the accumulation of amides. The loss would account for between 20 and 40 % of the total production of carbon dioxide over the corresponding period of starvation. There can be little doubt from the evidence presented here and in earlier papers that an important part of the respiratory substrate of starved barley leaves is derived from cell proteins. The work described by Vickery *et al.* (1937) (1939), and Wood *et al.* (1943, 1944) give strong support to this conclusion. A breakdown of proteins in respiratory processes appears to be a general feature of the metabolism of starved leaves.

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